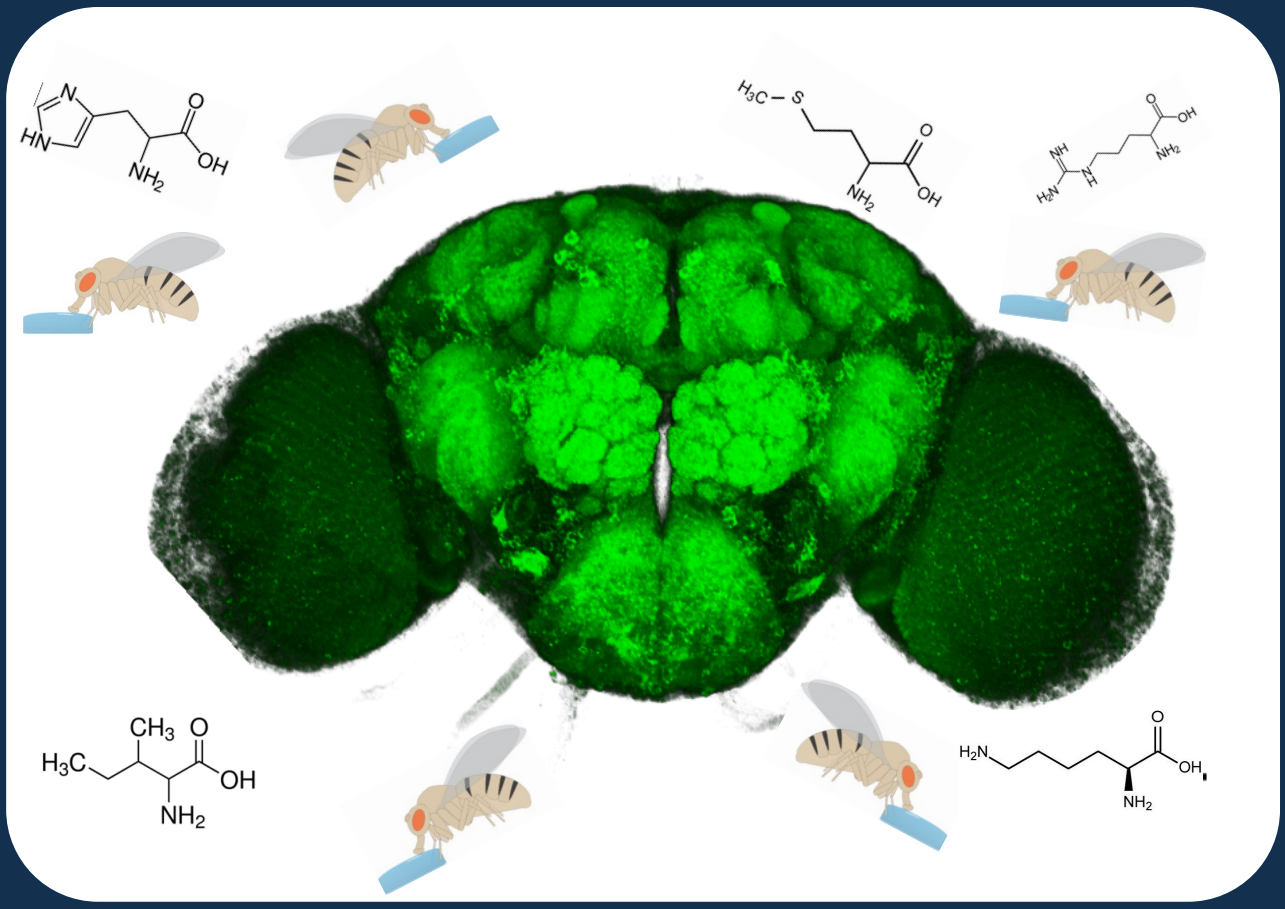
How the nervous system responds to and regulates amino acid homeostasis

Samantha L. Herbert



Dissertation presented to obtain the PhD degree in Biology | Neuroscience

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Lisbon,
September, 2017

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Research work coordinated by:

Fundação Champalimaud

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Acknowledgments

I would like to take this opportunity to thank everyone who has contributed to the research presented in this thesis – whether it was with new ideas, technically, or the 'occasional' emotional support!

In particular I would like to thank the following people:

Carlos Ribeiro, my supervisor, who welcomed me into the lab as the first PhD student many years ago. It has been a great learning experience! Thank you for sharing your knowledge, ideas and enthusiasm for science with me.

My thesis committee, Luísa Vasconcelos and Rui Costa for their yearly critiques.

The Behaviour and Metabolism lab, past and present: Ana Paula Elias (for magical cloning support), Célia Baltazar (teaching me the words to Parabéns a você, and how to do 2-choice colour assays), Pavel Itskov (introducing the wonderous flyPAD to the lab, and saving me from 15 hr fly sortings), Veronica Corrales (for her enthusiasm!), Gabriela Fioreze (for the fun and drinking), Ricardo Gonçalves (teaching me it's all about the right controls and the right n), Kathrin Steck (encouragement and optimism – after all we all love to present our new and exciting data), Zita Santos (teaching me how to do a western blot), Patrícia Francisco (always there when I needed), José Maria Moreira (always ready for a laugh), Samuel Walker (bringing a little piece of England to the lab), Margarida Anjos (helping me with anything – from fly work, to western blots to synthetic food

disasters), Dennis Goldschmidt and Daniel Münch (both new to the lab, but already doing new and exciting experiments!). Thank you all for this, and so much more!

The fly community for stimulating discussions, sharing reagents, and making the fly room the best room in the CCU – it's not all about the view!

The fly platform for the injections, the vast amounts of fly food they have made for me, and keeping a careful watch over the expiration date of my fly stocks.

All those at the CCU who have made this an inspiring, exciting and fun place to do science.

And finally, Alex – I could not have done it without you. Yes, fine, it's a bit cheesy, and probably one of the most over-used phrases in acknowledgements. However, in this case it is really true. You didn't only help or support, you were with me 100% of the way, and put up with the best and worst of it all! I really could not have done it without you.

I would also like to thank the members of my jury: Dr J Andrew Pospisilik (Max Planck Institute of Immunology and Epigenetics, Dr Alisson Gontijo (Chronic Diseases Research Center) and Dr Albino Oliveira-Maia and Dr Luisa Vasconcelos (Champalimaud Neuroscience Centre). Your questions were both insightful and probing, and I am very grateful to you all for your contributions!

So, thank you everyone, and here's to the next step!

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Chapter 1

1.1. Summary

For optimal growth, reproduction and lifespan an animal must eat a 'balanced' diet. This balanced diet is key in maintaining nutrient homeostasis in the animal. To achieve this balanced diet, the nervous system of an animal must have in place molecular mechanisms in order to both sense nutrient availability and change the feeding behaviour of the animal appropriately so it either selects or rejects particular foods. Whilst this is a rapidly growing field of research, the genes and cellular mechanisms underlying the translation of nutrient information into behavioural modification remains unknown. The work presented in this thesis first addresses the question of what constitutes a 'balanced diet'. I found that a diet in which the ratio of amino acids matches the exome may represent a balanced diet for the fly in terms of amino acid content. This diet is more satiating for flies, and they eat less of it, allowing early life history traits such as reproduction to be maximised without shortening lifespan. In addition, I asked what cellular pathways respond in an animal's nervous system if certain nutrients, in this case proteins or amino acids are missing in the diet. I find that mechanistic Target Of Rapamycin (mTOR) signalling and autophagy activity in head extracts of flies are correlated with the animal having eaten more or less dietary protein/amino acids. This suggests activity in these pathways responds in the nervous system in the face of nutrient challenges. I also investigate whether changes in neuronal mTOR and autophagic pathway activity play an instructive role in the

modulation of an animals' behaviour in response to a lacking nutrient. I find evidence that genetic manipulation of these pathways is not always sufficient to drive changes in food choice following a period of nutrient deprivation. Furthermore, I asked if the nervous system requires access to amino acids in order to effectively regulate behavioural changes. I find that the neuronal levels of arginine, an essential amino acid, fluctuate with dietary protein availability. I also identify a conserved putative amino acid transporter, I termed *beefeater* that is both necessary and sufficient in the nervous system to modulate food choice behaviour. Together, these results shine new light on our understanding of the genes and molecular pathways used by the nervous system of animals to modify food choice behaviour and maintain physiological levels of nutrients in the face of internal and environmental changes.

1.2. Sumário

Uma dieta equilibrada tem um papel decisivo na otimização do comportamento e sucesso de um animal. Esta terá de ser necessariamente uma dieta contendo o conjunto de macro- e micronutrientes que maximiza o crescimento do organismo, a progenia e esperança de vida.

Usando a *Drosophila melanogaster* como modelo animal, no primeiro capítulo da minha tese começo por abordar a questão do que é uma dieta equilibrada, para além da sua definição familiar, e proponho que, do ponto de vista proteico, esta

consiste numa combinação holídica de aminoácidos que replica essas mesmas proporções tal como são encontradas codificadas no ADN exómico do respetivo animal.
Partindo deste princípio, e usando variações à dieta, comprovei que é precisamente uma dieta dita 'exómica' aquela que sacia de forma mais completa os animais, e com menor quantidade de comida, permitindo maximizar os vários parâmetros de sucesso sem encurtar o seu tempo de vida – um equilíbrio fundamental e sempre discutido em estudos anteriores.

No seu habitat natural, a *Drosophila melanogaster*, ou mosca-do-vinagre, alimenta-se tanto dos açúcares da fruta em processo de fermentação como da levedura que efetiva essa transformação química. Embora saibamos que é desta forma que a mosca-do-vinagre consegue nutrir-se de proteínas e hidratos de carbono numa proporção que otimiza os seus parâmetros de sucesso vitais, os mecanismos moleculares que regulam esta homeostase e o comportamento animal que a controla ainda são desconhecidos.

Para um animal poder decidir o que come, quando come, ou quanto necessita de comer, o seu sistema nervoso tem de conseguir detetar alterações do estado nutricional do organismo e usar essa informação de modo a condicionar as escolhas de alimentação. No capítulo 4, abordei essa questão observando como o sistema nervoso da mosca-do-vinagre responde à omissão de um nutriente específico, neste caso uma proteína ou um aminoácido, na sua dieta. Comecei por otimizar vários métodos de quantificação de atividade de diferentes vias de sinalização celulares no sistema nervoso e descobri que tanto a

sinalização mTOR (mechanistic Target Of Rapamycin) como a atividade autofágica respondem de forma consistente e correlacionada a uma dieta deficiente em proteínas ou aminoácidos (diminuição em mTOR; aumento na autofagia).

Por outro lado, também foi possível determinar uma correlação entre os níveis do aminoácido essencial arginina e a falta de proteína numa dieta. Usando este indicador, questionei se a atividade da via autofágica desempenha um papel dirigente na modulação do comportamento animal em resposta à deficiência nutreica (capítulo 5).

Estes resultados sugerem que os aminoácidos neuronais têm uma função orientadora dos hábitos e comportamento alimentar. Em particular, consegui identificar um gene, anotado até agora como *CG12531* e à qual dei o nome de *beefeater,* bem conservado entre espécies (capítulo 6). *beefeater* codifica uma proteína com função prevista de transportadora de aminoácidos. Aqui demonstrei que a Beefeater é não só necessária como suficiente para a regulação do comportamento alimentar pelo sistema nervoso. A identificação deste novo mecanismo de regulação sugere que o sistema nervoso necessita aceder internamente aos aminoácidos para atualizar o comportamento alimentar do animal de acordo com a aferição dos níveis internos de nutrientes.

Finalmente, e no seu conjunto, estes resultados abrem uma nova perspetiva nova sobre as formas de regulação alimentar e apetite, mostrando a existência de vias moleculares homeostáticas usadas pelo sistema nervoso dos animais para modificar o comportamento alimentar e assegurar a

manuntenção dos níveis fisiológicos de nutrientes de acordo com mudanças internas e de ambiente.

Chapter 2. Introduction

2.1. Homeostatic feeding behaviour

The term homeostasis was coined in the early 20th century by Walter Cannon and popularised in his book *The Wisdom of the Body* [1]. He used the word to describe the maintenance of various steady states within the body, and the physiological processes through which they are regulated. Nutrient homeostasis, such as amino acid homeostasis, assumes that animals have an optimal internal nutrient availability, critical for both health and wellbeing. Whilst it is possible for an animal's internal storage mechanisms to compensate for times when nutrients are abundant or scarce, the selection or rejection of nutrients, or 'feeding behaviour', is ultimately the key mechanism an animal uses to regulate nutrient homeostasis [2].

The signalling molecules regulating an animal's feeding behaviour and nutrient homeostasis may be found within the food itself, or be generated by the animal's metabolic pathways. Proteins, fats and sugars make up a large proportion of the food animals consume. These macromolecules are broken-down by evolutionarily conserved biochemical pathways. The end products of these pathways include energy and other 'building blocks' of the organism such as amino acids. It is the nutrients themselves or the by-products or end products of the animal's biochemical pathways that may signal to the animal whether a specific nutrient is available or not. However, the exact nature of these signalling mechanisms remains unknown and is the subject of an intense field of research [3]. In particular, given the

important role of the nervous system in behavioural regulation, it is of general interest to determine how nutrient availability is sensed by the nervous system and interpreted in order to regulate nutrient-specific appetites. Understanding this will help understand not only how animals regulate nutrient homeostasis, but, how they might live healthier and longer lives.

The nervous system can regulate feeding behaviour at the level of both the peripheral (taste and smell) and central nervous system [3]. The nervous system also controls feeding behaviour over a range of timescales, controlling the initiation of a meal, termination of a meal, and can even determine what an animal will eat days into the future [3].

2.2. The regulation of feeding behaviour by the nervous system

The senses, primarily smell and taste, are known to play an important role in food selection. Both the olfactory and gustatory systems confer the recognition and discrimination of a large number of structurally distinct chemical molecules. The perception of these molecules is essential for the animal to identify relevant food sources as well as recognise palatability, and avoid harmful food sources.

The olfactory system of *Drosophila* consists of olfactory sense organs called sensilla, found on the antennae and maxillary palps. The olfactory receptor neurons (ORNs) extend dendrites into these sensilla. *Drosophila* has approximately fifty different

types of ORN, each expressing a set of olfactory receptors or in exceptional cases, receptors of the gustatory receptor gene family [4]. ORNs expressing the same receptor converge on the same glomeruli, dense neuropile structures in the antennal lobe. Within each glomerulus, ORNs form synapses with projection neurons (PNs) and a network of local interneurons. Approximately 180 PNs project to the mushroom bodies (MB), which are thought to be mainly involved in the formation of conditioned responses to odours, and to the lateral horn (LH), which is thought to mainly mediate innate responses to odours.

The gustatory system of *Drosophila* is also organised into sensilla, which are distributed in various organs in the fly body, including the labellum at the distal tip of the proboscis, the distal tarsal segments of the legs, and pharyngeal organs lining the oesophagus, all of which regulate feeding behaviours [5]. Gustatory neurons have been described as responding to sweet, water, low salt, bitter and high salt. In addition to these taste categories, a group of neurons in *Drosophila* has been identified in the proboscis labellum as responding to carbonated water, and mediating taste acceptance behaviour [6]. Finally *Drosophila* has shown proboscis extension responses following labellar stimulation with pure amino acid solutions and is thought to be able to detect amino acids at the level of the sensory neurons [7].

The assessment of the food after the animal has ingested it is known as post-ingestive assessment. Studies on energy homeostasis found that animals can select metabolisable carbohydrates over non-metabolisable carbohydrates, independent of their sensory properties [8]. Furthermore, the formation of stable associative memories requires exposure to

metabolisable carbohydrates, while the sensory properties of that sugar are secondary to the reinforcement [9, 10, 11]. Studies focused on amino acids found that animals, including *Drosophila* larvae, can detect if they are eating a meal lacking in a single essential amino acid, and reject it [12].

Finally, the feeding behaviour of animals can be driven over a period of days by changing 'internal states'. For example, *Drosophila* that have been deprived of protein for three days (and only fed sucrose) show an increased preference to eat yeast when given the choice between yeast and sucrose [13]. These flies are thought to be eating in order to replenish the missing protein.

All levels of control – immediate, post-ingestive or internal-state driven, allow an animal to effectively search out and find food sources that are not harmful and contain the nutrients that are currently missing. An increasing amount of work has been done characterising the sensory neurons, their responses and where the neurons project. However, concerning the cellular mechanisms and genetic underpinning of nutrient sensing at these various levels of feeding behaviour regulation much remains unknown.

2.3. Behaviour and genetics

The search to understand behaviour in terms of genetics and cell biology encompasses some of the most exciting questions in biology. The field of 'behavioural genetics' arguably starts with

the 19th century work of Galton, who studied the heritability of human abilities and mental characteristics [14]. However, his work took him down the treacherous path of eugenics, discrediting much of this emerging area of research. The field only regained a positive status following the publication of *Behavior Genetics* by Fuller and Thompson, a comprehensive review and critique of human behavioural genetics [15]. Since then the field of behavioural genetics has grown immensely [16]. It is now widely accepted that behavioural traits and disorders are indeed influenced by genes as well as environmental factors.

Work in *Drosophila* has been at the forefront of behavioural genetics research since the beginning, and has provided many important insights into the molecular, cellular and evolutionary bases of behaviour. In 1961, Hirsh used artificial selection on natural populations to alter the upward or downward movement of flies walking in a vertical maze [17]. He showed that there is a genetic basis for geotactic responses in flies. Later, the question was also raised as to whether the heritability of a behavioural trait could be linked to a single gene. Consistent with this hypothesis, Bastock showed that mutations in a single gene, *yellow*, could affect courtship in flies [18]. Following this, Seymour Benzer, William Park, Martin Heisenberg and their labs used forward genetics, and screened randomly induced mutations in *Drosophila* for behavioural phenotypes. These two approaches of behavioural selection and mutation fuelled the growing field of behavioural genetics and continue to inspire research. Today, *Drosophila* has successfully been used to identify how genes contribute to foraging and feeding, circadian, courtship, learning and memory behaviours [19]. Furthermore, *Drosophila* only has

around 200,000 neurons, thus simplifying the disentangling of genetics, molecular mechanisms and behaviour. From this research, it has been possible to derive lessons of general significance to the question of how genes affect complex behaviours.

Regarding the regulation of feeding behaviour, the use of *Drosophila* allows one to ask with relative ease what genes are involved in the regulation of the nervous system in response to nutritional information, and furthermore, how these genes act in a nutritionally dependent way in neurons to modify feeding behaviour.

2.4. The role of protein in controlling feeding behaviour

Whilst an animal's diet contains a mix, generally speaking, of the macronutrients, carbohydrates, proteins and fats as well as micronutrients, current experiments indicate that it is protein that has an especially potent role in driving behavioural change. Moreover, protein has been shown to have the greatest satiating effect of all the macronutrients [20]. This could derive from the fact that the essential amino acids found in proteins have to be eaten by the animal as they cannot be synthesised. This is a characteristic trait of all metazoans [21].

There is growing list of evidence describing the key role protein has in determining the food intake of an animal [13]. When *Drosophila* are given access to diets of varying protein:carbohydrate (P:C) ratios, the animals chose to eat a diet

of a ratio that maximises their lifetime egg production [22]. In addition, evidence from studies conducted in the wild, showed that animals can vary the foods they eat in order to maintain a specific P:C ratio when in different habitats. For example, a study on Ugandan mountain gorillas of the same species, but living in two different national parks, where the food sources vary, showed that these gorillas consume varying amounts of different foods, thus achieving diets of the same nutrient composition [23]. Importantly, when animals are faced with low P:C ratio foods, they tend to overeat carbohydrate in order to reach their protein target amount. This can have adverse effects for the animal, and has been hypothesised as contributing to the current obesity crisis in humans, it is termed the "protein leverage hypothesis" [24].

The question of how the nervous system senses changing internal levels of proteins and, in turn, how the animal responds behaviourally to changes in protein availability is highly relevant in the field of feeding behaviour. Detection of amino acids and control of protein intake takes place both peripherally, along the gastrointestinal tract and in the central nervous system of the animal [25]. In the periphery, the oral cavity of mammals contains several taste receptors for amino acids, and the labellum of *Drosophila* has been shown to be sensitive to amino acids, initiating an extension when stimulated with a mix of single amino acids [7]. The stomach of mammals contains 'stretch' receptors that can signal satiety. In addition, mammals secrete multiple hormones all the way along the gastrointestinal tract that are detected by vagal afferents that transmit the nutritional information to the central nervous system. In addition to these circulating peptides acting in and on the nervous system to

control feeding behaviour, amino acids can act directly on the CNS to control feeding behaviour. Bjordal and colleagues showed that application of amino acids to an ex vivo *Drosophila* larval brain could stimulate calcium responses in a few dopamine neurons, and that these dopamine neurons are necessary to mediate the rejection of amino acid imbalanced food [12].

For both peripheral and central amino acid detection, cellular 'nutrient sensing' mechanisms must exist that can sense changes in amino acid availability. The mechanistic Target Of Rapamycin (mTOR) pathway and General Control Nonderepressing 2 (GCN2) pathway are both highly conserved pathways capable of sensing cellular amino acid availability. Given the importance of the nervous system in the control of behaviour, it is particularly interesting to note that there is evidence for both of these pathways playing a role in the nervous system in the regulation of feeding behaviour [13, 26]. Furthermore, in the nervous system these nutrient sensing pathways could represent a way of linking nutritional state to cellular changes that in turn modulate behaviour [3, 27].

2.5. Nutrient sensing pathways

Nutrient sensing pathways are common throughout evolutionary history. All cells, whether as unicellular organisms, such as yeast, or as part of multicellular organisms (e.g., humans), must possess mechanisms capable of transducing nutrient signals into cellular changes. These mechanisms coordinate nutrient sensitive processes such as growth and survival. However,

there is an emerging concept that these pathways could be used not only to help in cell growth and survival, but in the survival of the organism: in the same way as these pathways control transcription in response to amino acid deprivation to help the cell survive, they could also be helping in the control of feeding behaviour upon amino acid deprivation, to help the organism survive. At least two highly conserved pathways, the general control nonderepressing 2 (GCN2) pathway and the mechanistic Target Of Rapamycin (mTOR) pathway, have been described to be involved in the control of feeding behaviour [26, 13].

2.5.1. The General Control Nonderepressing 2 (GCN2) pathway

GCN2 is a multidomain protein that contains regions homologous to histidyl-tRNA synthetases juxtaposed to a kinase domain [28]. Activation of this kinase occurs in response to starvation of many amino acids and thus has been termed the 'general control' of amino acid biosynthesis. Originally identified in yeast, orthologues have since been found in mammals and *Drosophila (dGCN2)* [29, 28, 30].

In the earliest steps leading to the initiation of mRNA translation, amino acids are acylated (charged) to transfer ribonucleic acid (tRNA) by their cognate amino acyl tRNA synthetases. Following a period of starvation from essential amino acids the cognate tRNA is deacylated (uncharged). GCN2 binds multiple deacetylated tRNAs and in this activated state, dimerises and autophosphorylates [31]. The activated kinase phosphorylates the

alpha subunit of eukaryotic initiation factor 2 (eIF2α), a pivotal factor in the control of the initiation of translation in protein synthesis. Phosphorylation of eIF2α effectively slows the assembly of ribosomes onto mRNA, reducing protein synthesis, but favouring the translation of a few mRNAs with special features in their 5' leader sequence, encoding transcription factors such as ATF4, which serve to reconfigure gene expression and allow the cell to respond appropriately to its environment [32].

A role for GCN2 in feeding behaviour

Essential amino acids, by definition, have to be eaten, as the organism cannot synthesise them. Therefore, recognising when a diet is deficient in essential amino acids is of the utmost importance for survival in all metazoans. In 2005, two papers proposed GCN2 as a neuronal amino acid sensing mechanism to detect amino acid imbalanced food [26, 33]. According to these reports, the consumption of food lacking a single essential amino acid leads to the development of an amino-acid imbalance in the anterior piriform cortex (APC), which is sensed by the protein kinase GCN2, enabling animals to reject the EAA-deficient food. Hao and colleagues showed that directly inhibiting tRNA acylation in the APC by injecting alcohol derivatives of amino acids could inhibit feeding in animals specifically on a diet with basal levels of that amino acid. Furthermore GCN2-/- mice fail to recognise a diet deficient in a single amino acid, and do not reduce their consumption [26]. Maurin and colleagues also showed that mice would, over time, eat less of an amino acid imbalanced chow, and this response is blunted in $GCN2^{-/-}$ mutant mice. Moreover, the loss of GCN2 reduced levels eIF2α

phosphorylation, a target of the GCN2 kinase. In *Drosophila,* it was shown that larvae expressing a constitutively active form of GCN2 in dopaminergic cells were anorexic, they did not grow and eventually died from starvation [12, 34]. However, the GCN2-dependent model of specific amino acid sensing has recently been criticised in work by Zachary Knight. He found that whilst mice ate less of a diet devoid of all amino acids, there was no evidence that mice ate less of a diet deficient in 1 or 2 amino acids. Furthermore, in $GCN2^{-/-}$ mice, no feeding phenotype could be found in any feeding assay [35]. It is possible these contradictory results stem from differences in the animals prefeeding conditions, however, Knights data clearly shows that this mechanism is not nearly as robust or universal as has been implied by the existing literature, and suggest we may need to rethink the role of GCN2 in feeding behaviour.

2.5.2. The mechanistic Target Of Rapamycin (mTOR) pathway

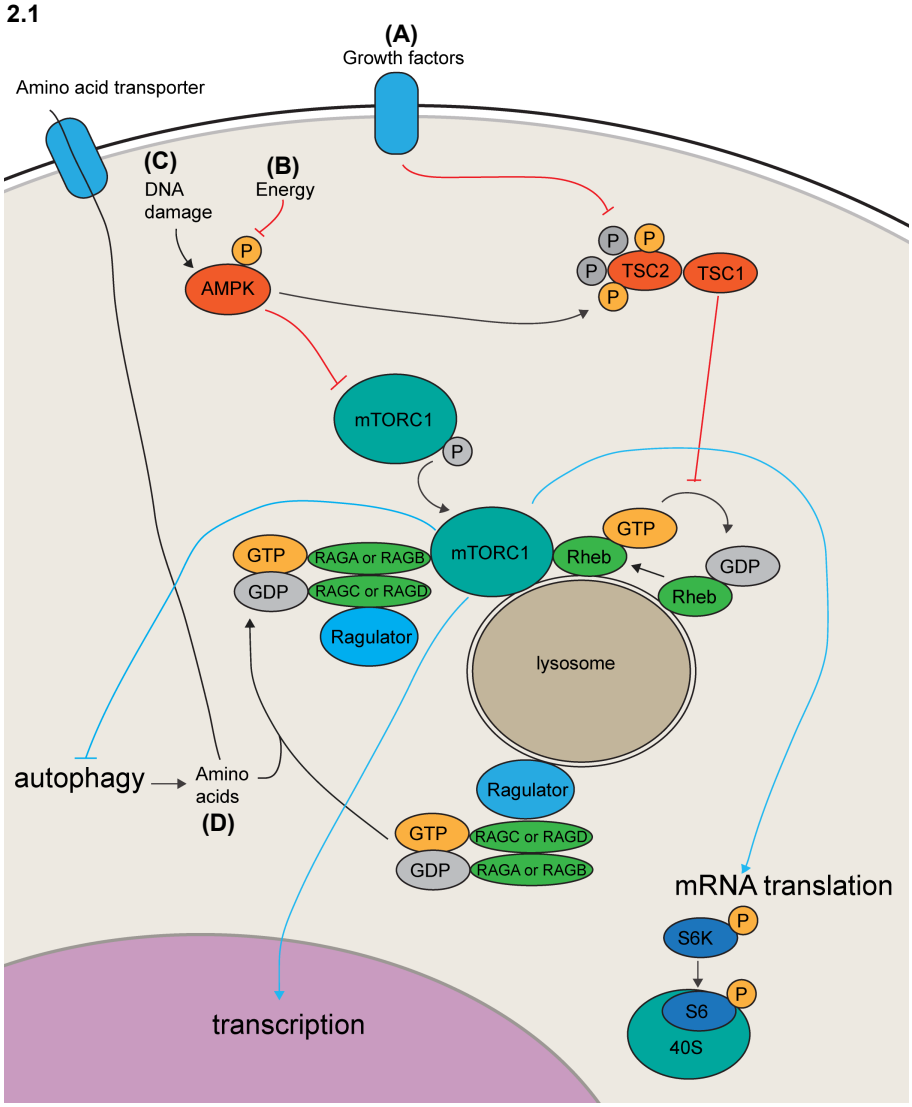
The protein mTOR was originally identified through mutations in budding yeast that confer resistance to the growth inhibiting properties of a bacterial metabolite, rapamycin [36]. It is a highly conserved protein, and to date every eukaryote genome examined contains a *TOR* gene. Eukaryote TORs are large proteins (approx. 280kDa), and belong to the phosphatidylinositol kinase-related kinase (PIKK) family. PIKK members have a carboxy-terminal serine/threonine protein kinase domain, and an amino-terminal made up of multiple domains for protein-protein interactions. This large multidomain protein exists in two distinct

complexes mTORC1 and mTORC2. Here I will focus only on the role and regulation of mTORC1.

One of the central roles of mTOR is as a controller of cell growth. Cell growth is an extensively coordinated process, regulated in both time and space. In yeast, when growth conditions are favourable, mTOR is active and the cells maintain a robust rate of ribosome biogenesis, transcription, translation initiation and nutrient import [37]. However, treatments with rapamycin or limiting nutritional conditions lead to a dramatic down-regulation of all these processes, and an up-regulation of (macro)autophagy (a cellular degradation process). Thus, mTOR signalling balances anabolic and catabolic processes depending on cellular context.

In multicellular organisms, the extracellular stimuli that are important for the activation of mTORC1 are hormones and growth factors, energy, stress and nutrients [37] **(Figure 2.1)**. The mTOR pathway responds to insulin or insulin like growth factors via the PI3K pathway, wired in by the tuberous sclerosis proteins TSC1 and TSC2. TSC2 acts as a GTPase-activating protein (GAP) for Rheb [38], and Rheb binds directly to the kinase domain of mTOR activating it in a GTP-dependent manner [39]. mTOR senses energy status of a cell through AMP activated protein kinase (AMPK). AMPK is activated in response to low cellular energy (high AMP:ATP ratio). AMPK directly phosphorylates TSC2, enhancing its GAP activity and inhibiting mTOR signalling [40]. Stresses, for example hypoxia and DNA damage are both interlinked with the AMPK signalling branch of the mTOR pathway [41, 42], activating AMPK to phosphorylate TSC2 to inhibit mTOR. Finally, nutrients, especially branched chain amino acids

2.1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Amino acid transporter
(C)
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damage
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Energy
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AMPK
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P
S6
40S
transcription

Figure 2.1. The mTOR signalling pathway. Mechanistic Target Of Rapamycin Complex 1 (mTORC1) promotes mRNA translation and transcription, and inhibits autophagy (blue arrows), by integrating nutrient signals such as amino acids, energy status, and growth factor information. **(A)** Insulin or insulin like growth factors activate the tuberous sclerosis proteins TSC1 and TCS2. TSC2 acts as a GTPase-activating protein (GAP) for the small GTPase Rheb. Rheb binds directly to the kinase domain of mTOR activating it in a GTP-dependent manner. **(B)** Energy status of the cell is sensed through AMP-activated protein kinase (AMPK). AMPK directly phosphorylates TSC2, enhancing its GAP activity and inhibiting mTOR signalling. **(C)** Stresses, for example DNA damage are also interlinked with the AMPK signalling branch inhibiting mTOR signalling. **(D)** Amino acids are proposed to activate mTOR via the Rag-Ragulator complex, which recruits mTOR to the lysosomal surface where is can be activated. Rag GTPases are heterodimers of either RAGA or RAGB with either RAGC or RAGD; the two members of the heterodimer have opposite nucleotide loading states. In the absence of amino acids, the Rag GTPases are found in an inactive conformation, amino acids cause a switch to an active conformation, causing mTORC1 to cluster onto the surface of late endosomes and lysosomes. Activating and inhibiting phosphorylations are orange and grey, respectively.

(e.g. leucine and arginine) regulate mTOR signalling [43, 44]. Amino acids are proposed to activate mTOR via the Rag-Ragulator complex, which recruits mTOR to the lysosomal surface. Rag GTPases are heterodimers of either RAGA or RAGB with either RAGC or RAGD; the two members of the heterodimer have opposite nucleotide loading states. In the absence of amino acids, the Rag GTPases are found in an inactive conformation, amino acids cause a switch to an active conformation that physically interacts with RAPTOR, causing mTORC1 to cluster onto the surface of late endosomes and lysosomes.

A role for mTOR in feeding behaviour

Whilst the appearance of mTOR signalling in unicellular organisms allowed them to sense nutrient availability and promote growth accordingly, with the emergence of multicellularity, mTOR acquired additional roles as a central controller of organism growth and homeostasis. For example, mTOR controls the growth not only of the cells it is active in, but also the growth of distant cells, and is involved in organ and an organisms size [45]. Furthermore, mammalian deregulated mTOR is implicated in disease states where growth is deregulated and homeostasis is compromised, including cancer, metabolic diseases and aging [46]. Interestingly, partial inhibition of mTOR function in yeast, worms, and flies, results in a significant increase in lifespan of these organisms [46].

Of particular interest is the role mTOR plays in metabolic diseases. The mTOR pathway is markedly elevated in the liver and skeletal muscle of insulin resistant obese rats maintained on a high fat diet [47], whereas the absence of the downstream mTOR

target S6 kinase, protects against diet-induced obesity and enhances insulin sensitivity in mice [48]. Given these observations linking mTOR activity with both diabetes and obesity, a line of research was started based on the hypothesis that mTOR might be able to integrate cellular fuel and nutrient status with hormonal related signalling and use this information to regulate food intake in animals.

With a focus on energy status of the animal, Cota and colleagues were the first to show that phosphorylated mTOR and phosphorylated PS6 kinase (a downstream target of mTOR), overlapped in expression in regions of the mediobasal hypothalamus (MBH) known to regulate feeding in mammals. Furthermore, fasting increased the expression of these phosphorylated proteins, and intracerebroventricular administration of the amino acid L-leucine, not only stimulated mTOR activity in these areas, but also suppressed feeding in the animals. Suggesting that neuronal mTOR can control food intake [49, 50, 51]. Furthermore, Wu and colleagues also investigated a possible role for mTOR signalling in neurons for coupling physiological hunger signals with hunger driven behaviours. They found that expression of a dominant negative or constitutively active form of S6K, a downstream target of mTOR, in *Drosophila* neurons was sufficient to trigger or inhibit hunger driven feeding behaviours in larvae [52].

The above research was focused the role of mTOR in responding to starvation of the animal and in the control of total food intake of an animal. However, it is known that mTOR is capable of responding to both energy signals (through the AMPK branch) as well as amino acids (through the Rags and Ragulator).

Interestingly, experimental evidence indicates that amino acids are sufficient to mediate neuronal mTOR responses to starvation [49]. Given this, the question arises of whether mTOR can respond to more subtle change in an animal's diet, for instance, only changes in protein, and furthermore, whether mTOR activity can modify feeding choices in a nutrient specific way.

The first evidence for amino acid specific mTOR signalling came from work by Ribeiro and Dickson. Using adult *Drosophila*, and testing them in a 2-choice behavioural setup, where the animals choose to eat either sucrose or yeast, they found that genetic manipulation of mTOR activity in the nervous system could increase the yeast preference of these animals, indicating that that mTOR can modulate nutrient specific appetites [13].

All together, it is clear that fasting (depriving an animal of all nutrients and energy) can elicit changes in mTOR activity in the nervous system of animals. Furthermore, genetic manipulations of mTOR can elicit changes in feeding behaviour. What remains unknown is whether nutrient specific manipulations of an animal's diet, for example, only removing proteins or amino acids can elicit changes in mTOR activity in the nervous system, and furthermore, whether this in turn guides nutrient specific appetites in the animal.

Downstream of mTOR in the cell

As mentioned above, mTOR maintains the balance between anabolic and catabolic processes in the cell according to the cellular context – energy and nutrient availability, hormonal signal or stresses. It carries out this complex function by regulating several downstream pathways **(Figure 2.1)**.

One of the most canonical functions of mTOR is the regulation of translation. Among the targets for translational control are transcripts that contain a 5' tract of oligopyrimidine (TOP) sequence. However, the mechanism of this control is uncertain, in some tissues mTORC1 regulates translation via S6 kinase and 4E-BP [37], but this is definitely not the case for all tissues. A second role of mTOR is in the control of the transcription of many genes and gene programmes including those involved in metabolic and biosynthetic pathways. These genes are either up-regulated or down-regulated according to the cells requirements [53]. The mTOR controlled transcription factors include TIF1A, previously shown to regulate the syntheses of Pol I, essential for ribosome biogenesis and TFEB, an essential regulator of lysosome function. Thirdly, mTOR controls (macro)autophagy. Autophagy is a cellular degradation process that involves the enclosure of parts of the cytoplasm, proteins or organelles in a double membrane bound structure, called the autophagosome [54]. The autophagosome is targeted to endosomes and lysosomes, where the vesicles fuse, allowing the contents of the autophagosome to be degraded by the lysosomal enzymes. The degraded contents are in turn recovered for re-use by the cell.

The downstream targets of mTOR are interesting because they represent the effectors or the outputs of the mTOR pathway. Determining how mTOR activity might change the activity of different cellular pathways in neurons in order to elicit the changes seen in feeding behaviour of the animal is essential if we are to understand how mTOR regulates feeding behaviour. In all the studies so far, the step of determining exactly how

modulation in mTOR signalling might affect feeding behaviour i.e. what exactly is changed in the neuron, has not been answered. Autophagy represents one possible downstream pathway for fast and specific neuronal modifications that could result in behavioural changes.

2.5.3. A focus on autophagy

In the late 1950's, morphologists working in mammalian cells first recognised autophagosomes as a unique compartment related to the lysosome, and in 1963 Christian de Duve coined the term 'autophagy'. The word autophagy originating from the Greek words *auto-,* meaning "self", and *phagein*, meaning "to eat". Since then the field exploded resulting in the award of the Nobel Prize in Medicine in 2016 to Yoshinori Ohsumi. (Interestingly Christian de Duve was also awarded the Nobel Prize in Medicine in 1974 for the discovery of the lysosome).

Following the initial discovery in yeast of the autophagy related (Atg) genes [55], most of these gene products turn out to be evolutionarily conserved, and this group of proteins' works together to carry out autophagy. The first crucial event in autophagy is the induction or nucleation of the membrane that will become an autophagosome. This pre-autophagosomal structure (PAS, phagophore assembly site) has been shown to form at the ER-mitochondria contact site in mammalian cells [56]. This event is detectable by the recruitment of *ULK1* (*dAtg1*). The kinase activity of this gene is required for the recruitment of *VPS34* (*dPi3K59F*), *VPS16*, *Beclin-1* (*dAtg6*) and *Atg14*

(*CG11877*), to the phagophore as part of a nucleation complex for the phosphorylation of phosphatidylinositol (PtdIns) to phosphatidyl inositol (3) phosphate (PtdIns(3)P). The recruitment of *ATG12* (*dAtg12*) and *ATG16L* (*CG31033*) are then required for the conjugation of phosphatidylethanolamine (PE) to *LC3* (*dAtg8a*), which is essential for the expansion and closure of the autophagosome membrane. The mature autophagosome then becomes acidified after fusion with the lysosome, forming the autolysosome. Lysosome fusion with the autophagosome provides luminal acid hydrolases that degrade the captured proteins, lipids, carbohydrates, nucleic acids and organelles. The degraded contents of the autolysosome are then secreted back into the cytoplasm by lysosomal permeases to provide nutrients for the cell use under stress conditions [54, 57] (**Figure 2.2**).

It is known that autophagy is responsive to amino acid fluctuations, and that mTOR is one of the key upstream effectors of amino acid mediated autophagy [58, 59]. mTOR is a potent repressor of autophagy, this is by the direct repression of ULK1 (dAtg1), and inhibition of mTORC1 is sufficient to induce autophagy in the presence of nutrients.

Whilst autophagy was initially thought of as a general 'recycling' mechanism in the cell, a lot of work has now been published showing that autophagy can selectively degrade cellular components. This layer of selectivity is an important mechanism to modify the activity of a neuron. For example, it has been shown that the selective degradation of the protein *highwire* at *Drosophila* larval neuromuscular junctions can regulate synaptic plasticity [60]. In addition, in *C. elegans,* autophagy selectively degrades GABA receptors in postsynaptic compartments to

2.2

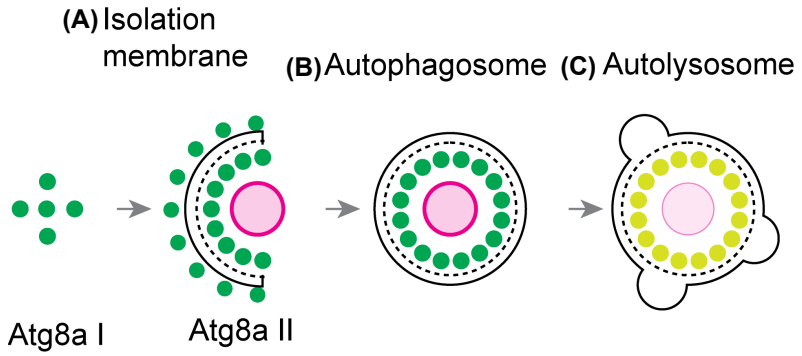


Figure 2.2. The autophagy pathway. **(A)** The induction or nucleation of the membrane that will become the autophagosome. **(B)** The conjugation of Atg8a to PE, and its subsequent relocation from the cytoplasm (Atg8a I) to the membrane (Atg8aII) is essential for the expansion and closure of the autophagosome membrane. **(C)** The mature autophagosome becomes acidified after fusion with lysosomes forming the autolysosome. The degraded contents of the autolysosome are released into the cell for re-use. Atg8a is shown as green circles, cellular content to be engulfed and then degraded is shown in pink, degraded Atg8a and cellular content is shown as yellow circles and light pink.

modify synapse strength [61]. The question raised in this thesis is whether autophagy responds in the nervous system to changing amino acid levels, and if so, what does it degrade that could modify the activity of the neurons and elicit changes in feeding behaviour?

2.6. The role of amino acid transport in amino acid homeostasis

Plants and fungi can synthesise each of the 20 amino acids using biosynthetic pathways inherited from their bacterial ancestors. However, the ability to synthesise nine amino acids (Phe, Trp, Ile, Leu, Val, Lys, His, Thr, and Met) was lost in a wide variety of eukaryotes that evolved the ability to feed on other organisms [21]. Eukaryotes therefore depend on amino acid transport not only for transport within their organism, but for transport into the body as well.

The availability of amino acids is recognised inside the cell by the nutrient responsive pathways GCN2 and mTOR. Amino acid transporters play a key upstream role as the 'gate-keepers' of the cell. The amino acids have to have access to the cell, or at least have a mechanism for being sensed inside the cell, in order for the downstream pathways to respond. There is a growing literature for amino acid transporters functioning as both transporters, or as 'transceptors', where they bind amino acids, upstream of nutrient sensitive pathways. Their role in feeding behaviour is also an area of increasing interest.

Amino acid transporters

In eukaryotic cells, amino acid transport relies on secondary transporters (i.e. transport that is not driven by the direct hydrolysis of ATP), and can be divided into either passive or active [62]. Passive transporters, also known as facilitated transporters, allow the passage of solutes across membranes down their electrochemical gradients. Active transporters on the other hand allow the passage of solutes across the cell membrane against their concentration gradient, in either exchange or symport. Transporters typically have a fixed stoichiometry of ion/solute movement per translocation cycle.

The Human Genome Organisation (HUGO) Nomenclature Committee Database provides a list of transporter families of the SoLute Carrier (SLC) gene series. A transporter was assigned to a particular family if it had at least 20-25% amino acid sequence identity to other members of that family. Currently this includes approximately 50 families, and in the human genome it is generally assumed that at least 5% of all human genes are transporter-related, consistent with the biological significance of transporters and their role in cell homeostasis [62]. 12 of these SLC families contribute at least one member to the transport of amino acids. 9 of these 12 are conserved in *Drosophila*. These include SLC1, 3, 6, 7, 15, 17, 32, 36 and 38 [63].

The SLC1 family of transporters transport glutamate and neutral amino acids [64]. They are essential for neurotransmission, as well as glutamate function outside the nervous system, where glutamate supports glutamine synthesis. SLC17 members have also been characterised as glutamate transporters at synapses [65].

The SLC6 family transports essential or conditionally essential amino acids, including those that can act as neurotransmitters [63]. Transporters of this family use the Na+ motive force to drive substrates across membranes. The SLC6 family can be divided into two large subfamilies, one mediating neurotransmitter transport, and the other, called the Nutrient Amino acid Transporters (NATs). The NATs have been linked with several metabolic and mental disorders.

The SLC7 family members play roles as basolateral or cell-specific permeases and exchangers of metabolic and nutrient amino acids [66]. SLC7 combines two major subfamilies, the first, the Cationic Amino acid Transporters (CATs), which have a 14 transmembrane domain (TMD) structure and differentially mediate transport of cationic amino acids Arg, Lys, and His. The second subfamily consists of the light subunits (L-type amino acid transporters (LATs)) of the heteromeric amino acid transporters (HATs). The light subunits form disulphide bridges with the heavy subunits from SLC3 to form HATs. Whilst CATs are mostly facilitated diffusers, the HATs are exchangers for a broad spectrum of amino acids.

SLC15 members have been characterised as proton driven oligopeptide and histidine transporters in the mammalian alimentary canal and other tissues [63]. OPT1 cloned from *Drosophila* has also been characterised as a broad substrate transporter [67].

The SLC32, 36 and 38 families are closely related, and thought to come from a common ancestor. They are also known as the β-group [68]. SLC32 and SLC38 are sodium coupled neutral

amino acid transporters (SNATs). SLC32 only has one member, which functions to pack inhibitory amino acids such as glycine or gamma-aminobutyric acid (GABA) into synaptic vesicles. SLC38 has multiple members that can be divided into system A and system N. These members vary as to where they are expressed, and how broadly expressed, with some members being tissue specific, and others expressed in almost every tissue tested [69]. SLC36 members function as proton coupled amino acid symporters [70]. The first cDNA corresponding to a member of the SLC36 family was isolated from rat brain and named LYAAT1 (lysosomal amino acid transporter 1) or PAT1 (proton coupled amino acid transporter 1). It is considered a low affinity, high capacity transporter with broad substrate specificity, including the amino acids glycine, proline and alanine. Other members, PAT2-4, have a higher affinity for their substrates. In general, this family is broadly expressed. *Pathetic,* an orthologue in *Drosophila,* has also recently been characterised [71].

Amino acid transporters upstream of mTOR

It was shown that Xenopus oocytes could respond to intracellular changes in amino acids by changes in mTOR signalling, such as the phosphorylation of the mTOR downstream target S6 kinase [72]. However, these cells did not respond to extracellular changes in amino acids unless expressing the SLC7 LAT member. These experiments strongly suggest this transporter, SLC7 LAT, is a conduit for the delivery of amino acids to an intracellular 'amino acids sensor' in the oocytes. Furthermore, a leucine analogue that blocks the SLC7 leucine transporter, has been shown to inhibit the stimulatory effects of leucine on S6K phosphorylation in rat cortical neurons [73]. In addition Nicklin and colleagues

identified 2 transporters necessary for the activation of mTOR, SLC1A5 for the influx of glutamine, and the heterodimeric SLC7A5/SLC3A2 bidirectional transporter, effluxing glutamine whilst importing leucine, which is necessary for the mTOR response [74].

There is increasing recognition that just the binding of particular amino acids to their respective transporter proteins could also serve as an effective means of sensing amino acid availability at the cell surface [75]. These proteins are known as 'transceptors'. The substrate/carrier-binding event must then be transduced to modulate an intracellular signalling response.

Ssy1 is one of the first examples of a transceptor that was found in yeast. Binding of amino acids is thought to trigger conformational changes in Ssy1, which are transduced via two transmembrane proteins to promote the N-terminal cleavage of two transcription factors, enabling their nuclear entry and promoting transcription [76]. In *Drosophila*, *CG3424/pathetic,* a gene related to the mammalian PATs has been proposed to act as a transceptor for mTOR signalling. The expression of this protein in Xenopus oocytes followed by amino acid flux quantifications, showed the protein to have a very low capacity for amino acid transport, but exceptionally high affinity for amino acids [71]. Given these transport characteristics the authors speculate that *pathetic* acts as a transceptor to modulate mTOR activity. Recently, two back-to-back studies identified SLC38A9 as a putative amino acid transceptor [77, 78]. This lysosomal membrane-resident protein was found to be competent of amino acid transport, albeit at low capacity. Moreover, its N-terminus

interacts physically with the Ragulator-RAG GTPases and signals amino acid availability to mTOR.

Amino acid transporters as regulators of feeding behaviour

The research detailed above shows that amino acid transporters are key in regulating amino acid homeostasis in animals. Furthermore, amino acid transporters have a direct impact on the nutrient sensitive pathways, including mTOR, with effects on growth of the animal. It is therefore an intriguing question as to whether there are amino acid transporters that may have key roles in regulating feeding behaviour of an animal.

2.7. Quantifying feeding behaviour in *Drosophila*

In order to determine the genetic contributions of the various nutrient sensing pathways to an animals behaviour it is necessary to have a robust behavioural paradigm. Behaviour is perhaps one of the most complex phenotypes to quantify. This stems from difficulties in defining what a particular behaviour is – even the simplest behaviour can be broken down into smaller individual behaviours. When determining if a behavioural phenotype is suitable for genetic analysis there are several points to take into account: is the behaviour robust; and is there a simple, easily reproducible way of quantifying that behaviour? *Drosophila* has emerged as a very powerful and genetically tractable organism in which to study the genetic underpinnings of feeding behaviour. This has relied heavily on the development of numerous feeding assays to quantify how much of a food a fly

eats and which food. These assays take into careful consideration the challenges derived from flies being so small and consuming such small quantities of food.

Traditionally, food labelling has been used to measure food intake in flies. In colourimetric assays the food source of interest is mixed with a coloured dye and left for the animal to feed on [79, 13]. Following a certain period of time, the assay is stopped and a quantitative assessment of how much the fly has eaten is made. This can be done by grinding up the fly and measuring the absorbance with a spectrophotometer. If an animal is being tested for a preference between two food sources, a qualitative assessment of the animal's food choice can be made by scoring the colour of the animal's abdomen, from this, a food choice index can be calculated. This is a simple assay, and is reasonably high throughput. It can quantify how much of a food source and what food a fly has eaten. For these reasons, I used this assay to quantify feeding on balanced and imbalanced diets (Chapter 3), to test the involvement of autophagy related genes (Chapter 5) and predicted amino acid transporters (Chapter 6) in food choice behaviour. The main draw back is that this assay cannot provide any temporal information about how the flies feeding behaviour progresses over time. Furthermore, there are some technical drawbacks, the assay does not account for excretion (dyes progress rapidly through the digestive tract) and so cannot be used for long assays. Furthermore, it can be difficult to find dyes that do not bias the flies feeding behaviour. An alternative is to use heavy isotopes instead of dyes. The quantity of food consumed can then be measured with a scintillation counter [80, 81]. This approach is more sensitive than

the colourimetric assay, but only allows for the indirect comparison of food preferences, so is more useful for measurements of absolute food intake and not comparisons between foods. Furthermore, the heavy isotopes are incorporated into the tissues of the animal, making ingestion and absorption difficult to separate.

One method that allows the dynamic measurement of food intake, as opposed to just the end-point, is the CApillary FEeding assay (CAFE). Flies consume liquid food from a graduated glass microcapillary. Descent of the meniscus allows continuous, unambiguous measurement of consumption. It is possible to monitor ingestion for periods ranging from minutes to an entire lifespan [82, 83]. However, flies are forced to eat upside down, which could affect their feeding habits, and the assay itself is reasonably laborious. Furthermore, yeast, a fly's natural source of protein is not a liquid, and so other substitute foods such as yeast extract must be used.

Capacitance or voltage based assays are the latest innovation in fly feeding quantification and circumvent most of the problems associated with the above assays. The fly Proboscis and Activity Detector (flyPAD) and Fly Liquid-Food Interaction Counter (FLIC) both use capacitive/voltage-based measurements to detect the physical interaction of individual flies with a food source [84,85]. Flies are put into arenas with wells of food, when the fly touches the food with its proboscis the capacitive/voltage signal is modified. Using the flyPAD it was found that the physical interaction of the fly's proboscis with the food is highly correlated with actual food intake. This interaction was therefore termed a 'sip'. Furthermore, it was found that these sips cluster into bursts

of sips spaced by various sized intervals. This was the first time a microstructure of feeding in flies had been documented. Capacitive/voltage-based assays allow for the quantification of how much food, what food, as well as a dynamic measurement of feeding, and all in a physiological setting. For these reasons, I used the flyPAD as a second behavioural assay to quantify food choice behaviour in flies when comparing feeding on balanced and imbalanced diets (Chapter 3) and when looking at the involvement of amino acid transporters in food choice (Chapter 6).

There are also assays monitoring feeding behaviours associated with food intake, for example, the Proboscis extension assay (PER). Upon stimulation of the gustatory receptors on the labellum or the tarsae, hungry flies will extend their proboscis if the substance is palatable, leading to the initiation of feeding [82]. Usually, the probability of extension of the proboscis is used as a quantitative measure in this assay. However, in this assay one must remember that although proboscis extensions always precede a meal, one can envisage that under certain circumstances it may not lead to food ingestion. Instead it tells you something about the keenness of the fly to eat.

Finally, in the last few years there has been a boom in quantitative approaches to behavioural analysis, giving rise to the field of computational ethology: the use of computerised tools to measure behaviour automatically, to characterise and describe it quantitatively, and to explore patterns which can explain the principles governing it [86]. This tackles head on the problems mentioned earlier associated with defining a behaviour – we no longer need to define it by eye, but careful monitoring and

quantification will tell us what the different aspects of the behaviour are. The use of such approaches in feeding behaviour has revolutionised it, and using video tracking approaches whole descriptions of foraging and feeding in naturalistic environments have been made [87].

2.8. Outline of work

Determining the genetic underpinnings of behaviour is not a simple task. The work of my doctoral thesis has focused on identifying and understanding the mechanisms of genes regulating amino acid specific appetite in *Drosophila* to control amino acid homeostasis. Specifically, I tackled the questions of what is the impact of a balanced diet on an animal's feeding behaviour? What nutrient sensing molecular pathways are activated or suppressed in the central nervous system in response to diets lacking in amino acids, and do these pathways modify the feeding behaviour of the animal? Does amino acid sensing in the nervous system play an important role in regulating these changes in feeding behaviour?

2.9. References

1. Cannon, W. *The wisdom of the body*. (New York: WW Norton and Company, inc, 1932).
2. Simpson, S. J. & Raubenheimer D. *The Nature of Nutrition*.
3. Itskov, P. M. & Ribeiro, C. The dilemmas of the gourmet fly: the molecular and neuronal mechanisms of feeding and nutrient decision making in Drosophila. *Front. Neurosci.* **7,** 12 (2013).

4. Fishilevich, E. & Vosshall, L. B. Genetic and functional subdivision of the Drosophila antennal lobe. *Curr. Biol.* **15,** 1548–1553 (2005).

5. Freeman, E. G. & Dahanukar, A. Molecular neurobiology of Drosophila taste. *Curr. Opin. Neurobiol.* **34,** 140–148 (2015).

6. Fischler, W., Kong, P., Marella, S. & Scott, K. The detection of carbonation by the Drosophila gustatory system. *Nature* **448,** 1054–1057 (2007).

7. Toshima, N. & Tanimura, T. Taste preference for amino acids is dependent on internal nutritional state in Drosophila melanogaster. *J. Exp. Biol.* **215,** 2827–2832 (2012).

8. Dus, M., Min, S., Keene, A. C., Young, G. & Suh, G. S. B. Taste-independent detection of the caloric content of sugar in Drosophila. *PNAS* **108,** 11644–11649 (2011).

9. de Araujo, I. E. *et al.* Food reward in the absence of taste receptor signaling. *Neuron* **57,** 930–41 (2008).

10. Burke, C. J. & Waddell, S. Remembering nutrient quality of sugar in Drosophila. *Curr. Biol.* **21,** 746–50 (2011).

11. Fujita, M. & Tanimura, T. Drosophila evaluates and learns the nutritional value of sugars. *Curr. Biol.* **21,** 751–5 (2011).

12. Bjordal, M. *et al.* Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in Drosophila. *Cell* **156,** 510–21 (2013).

13. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

14. Galton, F. *Hereditary Genius: An Inquiry into Its Laws and Consequences*. (London: MacMillan and Co., 1869).

15. Fuller, J. & Thompson, W. *Behavior Genetics*. (New York: John Wiley and Sons, 1960).

16. Ayorech, Z. *et al.* Publication Trends Over 55 Years of Behavioral Genetic Research. *Behav. Genet.* **46,** 603–607 (2016).

17. Hirsch, J. & Erlenmeyer-Kimling, L. Sign of taxis as a property of the genotype. *Science (80-.).* **134,** 1068–1069 (1961).

18. Bastock, M. A gene mutation which changes a behaviour pattern. *Evolution (N. Y).* **10,** 421–439 (1956).

19. Sokolowski, M. B. Drosophila: genetics meets behaviour. *Nat. Rev. Genet.* **2,** 879–90 (2001).

20. Bensaid, A. *et al.* Protein is more potent than carbohydrate for reducing appetite in rats. *Physiol. Behav.* **75,** 577–582 (2002).

21. Payne, S. H. & Loomis, W. F. Retention and Loss of Amino Acid Biosynthetic Pathways Based on Analysis of Whole-Genome Sequences Retention and Loss of Amino Acid Biosynthetic Pathways Based on Analysis of Whole-Genome Sequences. *Eukaryot. Cell* **5,** 1–6 (2006).

22. Lee, K. P. *et al.* Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proc. Natl. Acad. Sci.* **105,** 2498–2503 (2008).

23. Raubenheimer, D., Machovsky-Capuska, G. E., Chapman, C. A. & Rothman, J. M. Geometry of nutrition in field studies: an illustration using wild primates. *Oecologia* **177,** 223–234 (2015).

24. Simpson, S. J. & Raubenheimer, D. Obesity: the protein leverage hypothesis. *Obes. Rev.* **6,** 133–142 (2005).

25. Fromentin, G. *et al.* Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutr. Res. Rev.* **25,** 29–39 (2012).

26. Hao, S. *et al.* Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307,** 1776–8 (2005).

27. Pool, A. & Scott, K. Feeding regulation in Drosophila. *Curr. Opin. Neurobiol.*

28. Olsen, D., Jordan, B., Chen, D., Wek, R. & Cavener, D. Isolation of the gene encoding the drosophila melanogaster homolog of the saccharomyces cerevisiae

GCN2 eIF-2alpha kinase. *Genetics* **1045,** 1038–1045 (1998).

29. Santoyo, J., Alcade, J., Mendez, R., Pulido, D. & De haro, C. Cloning and characterization of a cDNA encoding a protein synthesis initiation factor-2a (eIF-2a) kinase from Drosophila melanogaster. *J. Biol. Chem.* **272,** 12544–12550 (1997).

30. Berlanga, J. J., Santoyo, J. & De Haro, C. Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2alpha kinase. *Eur. J. Biochem.* **265,** 754–762 (1999).

31. Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J. & Hinnebusch, a G. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. *Mol. Cell* **6,** 269–279 (2000).

32. Kang, M. J. *et al.* 4E-BP is a target of the GCN2 – ATF4 pathway during Drosophila development and aging. *J. Cell Biol.* **216,** (2016).

33. Maurin, A.-C. *et al.* The GCN2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. *Cell Metab.* **1,** 273–7 (2005).

34. Herbert, S. L. & Ribeiro, C. Nutrition: rejection is the fly's protection. *Curr. Biol.* **24,** R278-80 (2014).

35. Leib, D. E. & Knight, Z. A. Rapid Sensing of Dietary Amino Acid Deficiency Does Not Require GCN2. *Cell Rep.* **16,** 2051–2052 (2016).

36. Heitman, J., Movva, N. & Hall, M. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science (80-.).* **253,** 905–909 (1991).

37. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124,** 471–484 (2006).

38. Li, Y. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* **29,** 32–38 (2004).

39. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. Rheb binds and regulates the mTOR kinase. *Curr. Biol.*

15, 702–713 (2005).

40. Inoki, K., Zhu, T. & Guan, K.-L. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* **115,** 577–590 (2003).

41. Reiling, J. H. & Hafen, E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. *genes Dev.* 2879–2892 (2004). doi:10.1101/gad.322704.lating

42. Feng, Z., Zhang, H., Levine, A. J. & Jin, S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci.* **102,** 8204–8209 (2005).

43. Sancak, Y. *et al.* The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. *Science (80-.).* **320,** 1496–1501 (2008).

44. Sancak, Y. *et al.* Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141,** 290–303 (2010).

45. Colombani, J. *et al.* A nutrient sensor mechanism controls Drosophila growth. *Cell* **114,** 739–749 (2003).

46. Zoncu, R., Efeyan, A. & Sabatini, D. M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat. Rev. Mol. Cell Biol.* **12,** 21–35 (2011).

47. Khamzina, L., Veilleux, A., Bergeron, S. & Marette, A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. *Endocrinology* **146,** 1473–1481 (2005).

48. Hee Um, S. *et al.* Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431,** 485–485 (2004).

49. Cota, D. Hypothalamic mTOR Signaling Regulates Food Intake. *Sci. (New York, NY)* **312,** 927–930 (2006).

50. Blouet, C., Jo, Y.-H., Li, X. & Schwartz, G. J. Mediobasal Hypothalamic Leucine Sensing Regulates Food Intake through Activation of a Hypothalamus-Brainstem Circuit. *J.*

Neurosci. **29,** 8302–8311 (2009).

51. Blouet, C. & Schwartz, G. J. Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding. *Cell Metab.* **16,** 579–587 (2012).

52. Wu, Q., Zhang, Y., Xu, J. & Shen, P. Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila. *Proc. Natl. Acad. Sci.* **102,** 13289–13294 (2005).

53. Laplante, M. & Sabatini, D. M. Regulation of mTORC1 and its impact on gene expression at a glance. *J. Cell Sci.* **126,** 1713–1719 (2013).

54. Russell, R. C., Yuan, H.-X. & Guan, K.-L. Autophagy regulation by nutrient signaling. *Cell Res.* **24,** 42–57 (2014).

55. Tsukada, M. & Ohsumi, Y. Isolation and charactization of autophagy-defective mutants of. *Fed. Eur. Biochem. Soc.* **333,** 169–174 (1993).

56. Hamasaki, M. *et al.* Autophagosomes form at ER–mitochondria contact sites. *Nature* **495,** 389–393 (2013).

57. Zirin, J. & Perrimon, N. Drosophila as a model system to study autophagy. *Semin. Immunopathol.* 1–10 (2010).

58. Scott, R. C., Juhász, G. & Neufeld, T. P. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr. Biol.* **17,** 1–11 (2007).

59. Kamada, Y. *et al.* Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* **150,** 1507–1513 (2000).

60. Shen, W. & Ganetzky, B. Autophagy promotes synapse development in Drosophila. *J. Cell Biol.* **187,** 71–79 (2009).

61. Rowland, A. Presynaptic Terminals Independently Regulate Synaptic Clustering and Autophagy of GABAA Receptors in Caenorhabditis elegans. *J. Neurosci.* **26,** 1711–1720 (2006).

62. Hediger, M. a *et al.* The ABCs of solute carriers: physiological, pathological and therapeutic implications of

human membrane transport proteinsIntroduction. *Pflugers Arch.* **447,** 465–8 (2004).

63. Boudko, D. Y. Molecular basis of essential amino acid transport from studies of insect nutrient amino acid transporters of the SLC6 family (NAT-SLC6). *J. Insect Physiol.* **58,** 433–449 (2012).

64. Kanai, Y. *et al.* The SLC1 high-affinity glutamate and neutral amino acid transporter family. *Mol. Aspects Med.* **34,** 108–120 (2013).

65. Reimer, R. J. SLC17: A functionally diverse family of organic anion transporters. *Mol. Aspects Med.* **34,** 350–359 (2013).

66. Fotiadis, D., Kanai, Y. & Palacín, M. The SLC3 and SLC7 families of amino acid transporters q. *Mol. Aspects Med.* **34,** 139–158 (2013).

67. Roman, G., Meller, V., Wu, K. & Davis, R. The opt1 gene of Drosophila melanogaster encodes a proton-dependent dipeptide transporter. *Am. J. Physiol. Physiol.* 857–869 (1998).

68. Schiöth, H. B., Roshanbin, S., Hägglund, M. G. A. & Fredriksson, R. Evolutionary origin of amino acid transporter families SLC32 , SLC36 and SLC38 and physiological , pathological and therapeutic aspects. *Mol. Aspects Med.* **34,** 571–585 (2013).

69. Sundberg, B. E. *et al.* The evolutionary history and tissue mapping of amino acid transporters belonging to solute carrier families SLC32, SLC36, and SLC38. *J. Mol. Neurosci.* **35,** 179–93 (2008).

70. Thwaites, D. T. & Anderson, C. M. H. The SLC36 family of proton-coupled amino acid transporters and their potential role in drug transport. *Br. J. Pharmacol.* **164,** 1802–1816 (2011).

71. Goberdhan, D. C. I., Meredith, D., Boyd, C. a R. & Wilson, C. PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. *Development* **132,** 2365–2375 (2005).

72. Christie, G. R., Hajduch, E., Hundal, H. S., Proud, C. G. & Taylor, P. M. Intracellular sensing of amino acids in Xenopus laevis oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. *J. Biol. Chem.* **277,** 9952–9957 (2002).

73. Ishizuka, Y., Kakiya, N., Nawa, H. & Takei, N. Leucine induces phosphor- ylation and activation of p70S6K in cortical neurons via the system L amino acid transporter. *J. Neuchemistry* 934–942 (2008).

74. Nicklin, P. *et al.* Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell* **136,** 521–534 (2009).

75. Hundal, H. & Taylor, P. Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. *AJP Endocrinol. Metab.* **296,** E603–E613 (2009).

76. Forsberg, H. & Ljungdahl, P. Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. *Mol Cell Biol* **21,** 814–826 (2001).

77. Rebsamen, M. *et al.* SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature* **9,** (2015).

78. Wang, S. & Tsun, Z.-Y. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science (80-.).* **347,** (2015).

79. Tanimura, T., Isono, K., Takamura, T. & Shimada, I. Genetic Dimorphism in the Taste Sensitivity to Trehalose in Drosophila melanogaster. *J. Comp. Physiol.* **147,** 433–437 (1982).

80. Carvalho, G. B., Kapahi, P., Anderson, D. J. & Benzer, S. Allocrine Modulation of Feeding Behaviour by the Sex Peptide of Drosophila. *Curr. Biol.* **16,** 692–696 (2006).

81. Vargas, M. A., Luo, N., Yamaguchi, A. & Kapahi, P. A Role for S6 Kinase and Serotonin in Postmating Dietary Switch and Balance of Nutrients in D. melanogaster. *Curr. Biol.* **20,** 1006–1011 (2010).

82. V, D. *The Hungry Fly: A physiological Study of the Behaviour Associated with Feeding*. (Cambridge, MA: Harvard University Press, 1976).

83. Ja, W. W. *et al.* Prandiology of Drosophila and the CAFE assay. *Proc. Natl. Acad. Sci.* **104,** 8253–8256 (2007).

84. Itskov, P. M. *et al.* Automated monitoring and quantitative analysis of feeding behavior in Drosophila. *Nat. Commun.* 4560 (2014).

85. Ro, J., Harvanek, Z. M. & Pletcher, S. D. FLIC: High-throughput, continuous analysis of feeding behaviors in Drosophila. *PLoS One* **9,** (2014).

86. Anderson, D. J. & Perona, P. Toward a science of computational ethology. *Neuron* **84,** 18–31 (2014).

87. Corrales-Carvajal, V. M., Faisal, A. A. & Ribeiro, C. Internal states drive nutrient homeostasis by modulating exploration-exploitation trade-off. *Elife* **5,** 1–29 (2016).

Chapter 3. The effect of an exome matched diet on feeding behaviour

3.1. Summary

The question of what is a balanced diet has eluded researchers for many years. While it is clear that a balanced diet is one that will optimise the life history traits of an animal, what constitutes a balanced diet in terms of macro- and micronutrients remains unknown. In this chapter I outline work hypothesising that a balanced diet, at least in terms of essential amino acids, is one which matches the amino acid ratios found encoded in the exome. First, I determine the effects of this exome-matched diet on the feeding behaviour of the animal. I show that an exome-matched diet is more satiating for the fly, and that this is not simply a consequence of the animal eating more of it. I also show that the exome-matched diet is more appetitive than other, non-matched diets. These results indicate exome-matching may provide a theoretical framework for determining 'balanced' diets that can be applied to other organisms. The work presented in this chapter contributed significantly towards the publication of the research article, "Matching Dietary Amino Acid Balance to the In Silico-Translated Exome Optimizes Growth and Reproduction without Cost to lifespan" in the journal Cell Metabolism, of which I am a co-author [1].

3.2. Introduction

A 'balanced' diet is one that has all the nutrients needed for an animal's optimal growth, reproduction and lifespan. The term stresses that it is not just the amount of food that is critical for the animal's survival, but the ratio or balance between different nutrients.

There is mounting evidence that animals can balance their macronutrient intake to optimise the life history traits of growth, reproduction and lifespan [2]. It was shown in *Drosophila* that, when given access to different diets, flies eat to optimise their lifetime egg production. In choosing these specific diets *Drosophila* act like small "nutrient-seeking missiles" [3]. These diets varied in their protein: carbohydrate (P:C) ratio, with low P:C ratios leading to longer lifespans but reduced reproduction, and high P:C ratios resulting in shorter lifespans but higher rates of reproduction. Furthermore, evidence from studies conducted on animals in the wild showed they could vary the foods they eat to maintain a specific P:C ratio when faced with different availabilities of food [4]. This suggests wild animals may also be eating to optimise their life history traits, but this depends entirely on suitable food being available. When suitable foods are not available the animal may begin to suffer from malnutrition, starvation and even death. As an example, it has been proposed that the obesity crisis in humans is partly a response to many foods in our western diet having low P:C ratios, which would stimulate over-eating in order to reach protein target levels, the "protein leverage hypothesis" [5]. To explain this a little further, traditionally over-abundance of fat and carbohydrate have been

blamed as the greatest causes of obesity in humans. However, Simpson and Raubenheimer noticed that over many years, whilst the intake of fat and carbohydrate increased, protein intake stayed stable. They developed a model that shows how, paradoxically, it may be precisely because protein comprises such a tightly regulated component of the diet that it could have sufficient leverage over human ingestive behaviour to explain obesity. When an individual is faced with an imbalanced diet (relative to their individual intake target of protein and carbohydrate and fat), humans will prioritise protein, even if that means over-eating carbohydrate and fat.

Given that protein appears to be playing such a central role in determining feeding behaviour, I asked whether the quality of the protein source, as well as the quantity, might be important in determining the animal's consumption of that food. The quality of a protein is determined by its amino acid composition. Amino acid composition has already been implicated as important in the optimisation of reproduction and lifespan [6]. There is also research showing that animals can actively reject food if it is lacking in a particular amino acid, suggesting that animals have mechanisms in place to avoid foods of low protein quality [7, 8]. A hypothesis was developed where the quantification of coding regions of a genome, the exome, would be determinant to the establishment of the 'balanced' ratio of amino acids required by the animal, and as such the quality of the protein source. Specifically, using the *Drosophila melanogaster* genome, its predicted 19,736 protein-coding genes were translated, and from this the proportional representation of each of the 20 amino acids was derived. In this context, the development of holidic diets

(made entirely of purified chemical components) for *Drosophila* has been essential [9, 10]. They allow the minute dissection of the components of an animal's diet, such as the manipulation of amino acid ratios in the food, and the careful quantification of a multitude of physiological parameters of the animal.

I sought to answer the question of how an animal responds behaviourally to a food source with higher or lower protein quality using the exome of the animal as my guide. Previous work assessing the effects of protein quality on food intake have relied heavily on the use of oligidic (natural yeast based diet) or meridic (semi-chemically defined) diets, and the assumption that the manipulation of one or more amino acids creates an imbalanced food source [11]. The use of a holidic medium in this project allowed us to test if these findings are generalisable, and specifically, whether the effects seen in an animal's feeding behaviour are due to changes in one or two amino acids or alternatively, whether it is the change in the whole repertoire of amino acids that drives feeding behaviour. Finally, in comparing feeding behaviour on both exome-matched and un-matched diets we were able to test if exome-matching represents a quantifiable way of generating a balanced diet.

3.3. Material and Methods

Fly stocks: All experiments were conducted with female *Drosophila melanogaster* (Dahomey) or w^{1118} as indicated.

Holidic media: Holidic media were made according to Piper et al., 2014 [9] with appropriate substitutions for each of the amino acid ratios [1]. For the holidic media used in the MM1AA/FLYAA choice assay and for flyPAD experiments modifications to the diet included; agar was substituted for 1% agarose, added after autoclaving the food. The cholesterol was also added after autoclaving. No preservatives were used in this food.

Sugar/yeast choice assay: w^{1118} or Dahomey flies were reared in yeast-based food containing (per litre of water: 80g sugar cane molasses, 22g sugarbeet syrup, 8g agar, 80g corn flour, 10g soya flour, 18g yeast extract, 8ml propionic acid, 12 ml nipagin (15% in EtOH)). Upon emerging as adults, groups of 3-6 days old flies (15 females and 5 males) were transferred to fresh yeast based food or holidic medium. Two-choice colour feeding preference assays were performed as described in Ribeiro and Dickson, 2010 [12]. Flies were given the choice between sucrose mixed with red colourant (20mM sucrose; 7.5mg/ml agarose; 5mg/ml Erytrosin B (Sigma-Aldrich 198269); 10% PBS) and yeast mixed with blue colourant (10% yeast (SAF instant yeast); 7.5mg/ml agarose; 0.25mg/ml Indigo carmine (Sigma-Aldrich 131164); 10% PBS) medium. After visual inspection of the abdomen, each female fly was scored as having eaten sucrose (red abdomen), yeast (blue abdomen), or both (red and blue or purple abdomen) media. The yeast preference index (YPI) for the whole female population in the assay was calculated as follows: (nblue yeast – nred sucrose)/(nred sucrose + nblue yeast + nboth).

MM1AA/FLYAA choice assay: w^{1118} flies were reared as described in the SY choice assay. Upon emerging as adults,

groups of 3-6 days old flies (15 females and 5 males) were transferred to fresh yeast based food for 3 days followed by holidic medium for a further 3 days. Flies were given the choice between MM1AA mixed with red colourant (5mg/ml Erytrosin B Sigma-Aldrich 198269) and FLYAA mixed with blue colourant (0.25mg/ml Indigo carmine Sigma-Aldrich 131164). The FLYAA preference index (FLYPI) was scored as for the SY choice assay.

FlyPAD Monitoring of Feeding Behaviour: Dahomey flies were reared in the same medium as for the SY choice assay. Mated adult flies were then maintained on either holidic or yeast-based medium for the pre-treatment period and assayed using flyPAD, as described in Itskov et al., 2014 [13]. Where holidic medium was used the assay was conducted in the same way, but there was no choice, only 1 spot of food was made.

3.4. Results

3.4.1. An exome-matched diet is more satiating for the fly

In order to determine whether the exome defines the optimal nutritional protein requirement of an animal, I used a series of holidic diets - synthetic mediums made from entirely purified ingredients identical in every way, apart from the amino acid ratio **(Figure 3.1)**.

The first diet, FLYAA, was designed to match the exome of the fly. The entire coding region of the *Drosophila* genome was translated *in silico*, the number of each amino acid was

3.1

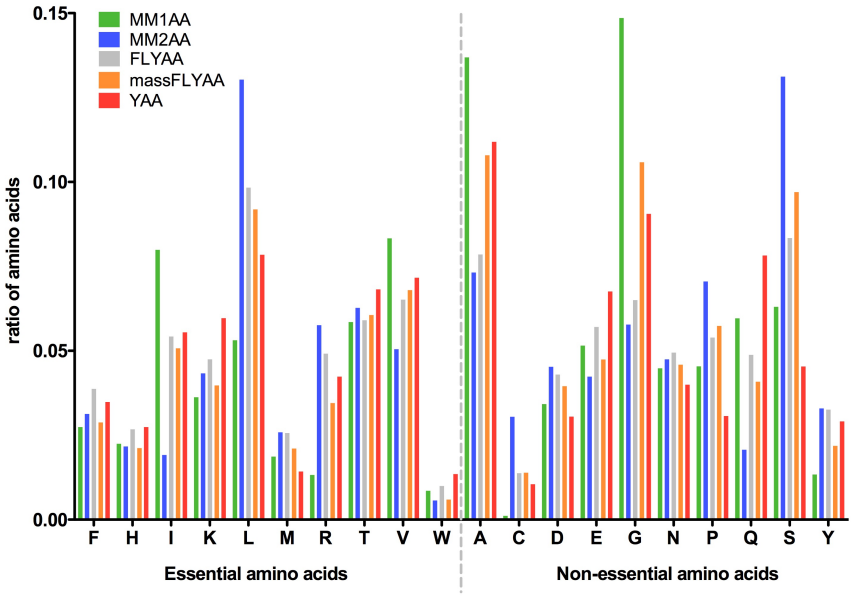


Figure 3.1. Comparison of the relative abundance of each amino acid (AA) profile used in this study. MM1AA (mismatch1 AA), MM2AA (mismatch2 AA), FLYAA (exome matched based on molar ratios of amino acids), massFLYAA (exome matched based on mass ratios of amino acids), YAA (yeast AA).

3.2

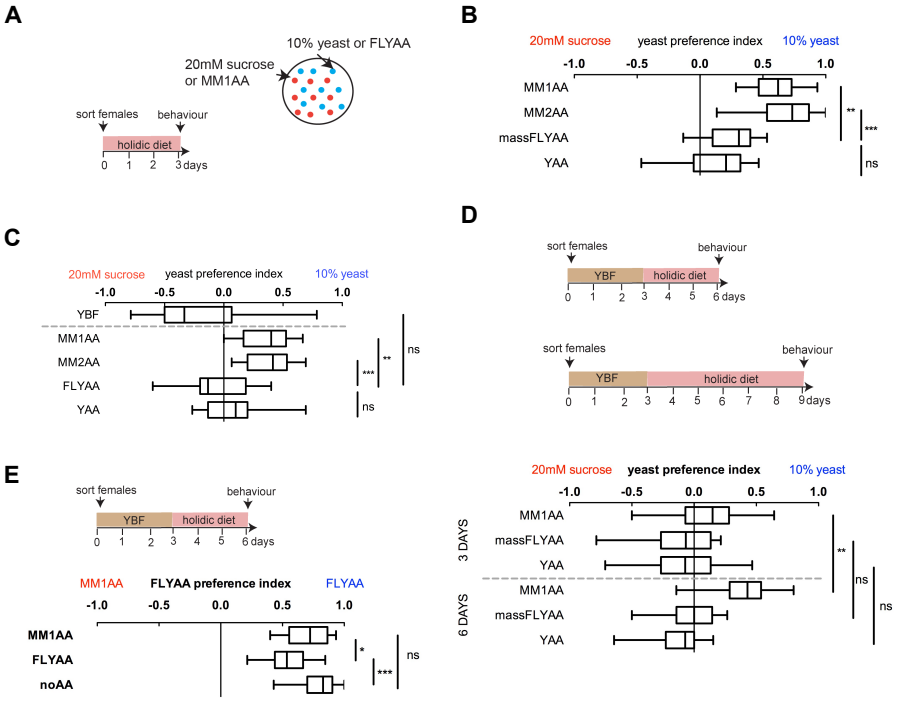


Figure 3.2. Exome-matched diets are more satiating. (A) Feeding pretreatment and 2-choice behavioural setup. **(B and D)** The yeast preference index (YPI) of w^{1118} flies or **(C)** Dahomey flies after pretreatment on each of the diets indicated down the left side of the graph. Feeding protocol is as in **(A)** unless otherwise specified. **(E)** The FLYAA preference index of w^{1118} flies after being prefed 3 days of YBF, followed by 3 days of the diets specified down the side of the graph. n=19-20 for all conditions; **(B and C)** Kruskal-Wallis test followed by Dunns multiple comparisons; **(D)** t-test; boxes show median and interquartile range, and whiskers show minimum/maximum values; p>0.05 ns, p<0.05*, p<0.01**, p<0.001***.

determined, and from that the molar ratio of one amino acid to another was calculated (an additional diet where the mass ratio was used instead was also used in some experiments and is referred to as massFLYAA). As a comparison diet, I used a previously published holidic diet [9] that was based on initial findings from Hunt and colleagues describing a diet on which fruit flies can live in the laboratory [14]. This diet is very different in terms of amino acid composition when compared to FLYAA, and is referred to as Mismatch 1 (MM1AA). To control for diet specific differences that might be due to the variation of one or more specific amino acids (and not the ratio between all of them), a second mismatched diet was used, Mismatch 2, MM2AA. Finally, another previously published holidic diet was used, matching the proteome of yeast, YAA [9].

If exome-matching does indeed provide a balanced diet, or high-quality protein source, in terms of amino acids, there are several predictions that might be made regarding changes in feeding behaviour. The first is that an animal having been fed this diet would be more satiated than an animal prefed a diet in which something was lacking. It is known that removal of amino acids, even single essential amino acids, from the diet is sufficient to increase the yeast preference of flies [15]. To test if this observation is also apparent following the prefeeding of flies with food of differing AA ratios, I pre-fed flies one of the five AA ratios, or yeast based food (YBF), and then used a 2-choice feeding assay in which flies are given the option of 10% yeast with blue food colouring and 20mM sucrose with red food colouring **(Figure 3.2A)**. The yeast preference index of these flies is calculated by scoring the colour of the flies' abdomen. In the first

instance, w^{1118} flies were tested, and the exome-matched diet (massFLYAA) effectively reduced yeast preference compared to both the non-exome matched diets (MM1AA and MM2AA). The yeast preference was comparable to that seen after keeping flies on a diet matching the proteome of yeast (YAA) **(Figure 3.2B)**. This was the same for Dahomey flies, where the exome matched diet (FLYAA) brought the levels of yeast preference down to those found following a diet of yeast based food (YBF) and a diet matching the proteome of yeast (YAA) **(Figure 3.2C)**. Interestingly, I noticed that the exome matched diet (massFLYAA) was also better at keeping the flies more satiated over a longer period of time. Increasing the number of days flies were kept on holidic medium from three to six after pre-feeding with YBF, increased the yeast preference of the flies fed a non-exome matched diet (MM1AA) compared to those fed an exome-matched diet (massFLYAA) or a yeast matched diet (YAA) **(Figure 3.2D)**. Furthermore, I asked if flies would show a similar satiation effect after being fed FLYAA if they were instead given a choice of MM1AA or FLYAA in a 2-choice behavioural assay. I found flies that had been prefed FLYAA exhibited a lower preference for FLYAA than flies previously fed a non-exome matched diet (MM1AA) or a diet that contained no amino acids (no AA). Supporting the conclusion that FLYAA keeps flies more fully fed, or satiated (**Figure 3.2E**).

Changes in yeast preference can be driven by modification to either yeast or sucrose feeding. To ask if these changes in yeast preference are driven specifically by changes in yeast feeding behaviour I used a single choice set-up in the Fly Proboscis and Activity Detector (FlyPAD) **(Figure 3.3A)**. This behavioural

3.3

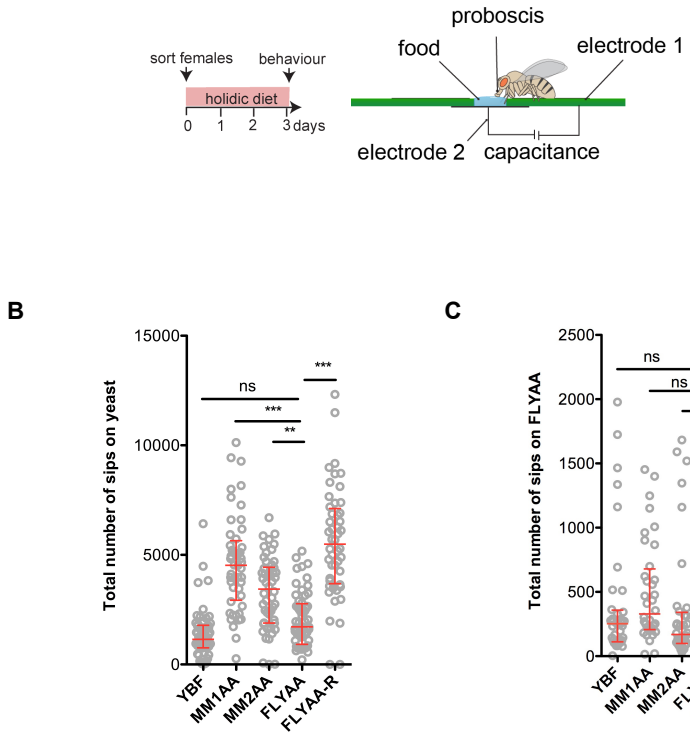


Figure 3.3. Exome-matched diets modify protein rich food consumption. (A) Prefeeding treatment and the flyPAD behavioural setup. **(B)** Total number of sips on yeast, **(C)** total number of sips on FLYAA of Dahomey flies, after being pretreated on the indicated diets shown on the x-axis. n=35-56 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; error bars represent the median and IQR; p>0.05 ns, p<0.01**, p<0.001***

monitoring system uses capacitive-based measurements to detect the physical interaction of individual flies with a food source, allowing for quantification of yeast feeding behaviour in a time dependent fashion. This physical interaction of the fly touching the food with its proboscis is highly correlated with actual food intake, and is termed a 'sip'. Dahomey flies that had been fed the exome-matched FLYAA had a reduced number of sips on yeast compared to non-exome matched diets (MM1AA and MM2AA), that was no different to the number of sips from flies kept on YBF. **(Figure 3.3B)**. Indicating that the changes seen in the preference index of flies fed the exome matched diet could be driven by changes in yeast feeding. I also included a pre-feeding condition where a single amino acid had been omitted from the food to show how similar this condition is to the conditions where non-exome-matched food had been given. When using FLYAA as the substrate instead of yeast on the flyPAD arena I saw no difference between flies that had been prefed FLYAA or mismatched diets **(Figure 3.3C)**. This could perhaps be due to the difference in strength of the food as a sensory stimulus; in general, the total number of sips was almost ten times lower than when yeast was given. However, the increase in number of sips seen on FLYAA following pretreatment with the FLYAA-R diet suggests the reduced number of sips is not contributing to the lack of phenotype seen, but instead suggests the two assays may be dependent on different underlying mechanisms.

The fact that the exome-matched food appears to be more satiating for the flies could stem from two factors. Perhaps the ratio of AAs in this food source can be utilised much more

effectively, so to achieve the same physiological effects the flies can afford to eat less. Alternatively, the flies could eat more of the exome-matched food – perhaps because it tastes better. To test this, I kept flies either on MM1AA or FLYAA for three days. I then gave the flies a single spot of the same food they had been kept on for three days, and quantified the total number of sips in the flyPAD setup. I found that flies consumed less FLYAA when compared to MM1AA **(Figure 3.4)**. This indicates that it is the ratio of amino acids in FLYAA, and not the amount consumed that leads to FLYAA being more satiating for the fly.

3.4.2. An exome-matched diet is more appetitive for the fly

The second hypothesis for how a 'balanced' diet might influence feeding behaviour is that the diet itself maybe be more appetitive. To test this I took flies and asked if they would have a higher number of sips on an exome-matched diet than on the mismatched diets. Using the flyPAD with single spots of the different diets I determined that flies prefed a yeast based diet (YBF) did indeed show a higher number of sips on FLYAA than on the two mismatched diets **(Figure 3.5A)**. This effect appears to be at least partially dependent on the internal state of the fly. Flies that had been previously deprived of dietary amino acids showed an increased number of sips on FLYAA compared to MM2AA, but not compared to MM1AA **(Figure 3.5B)**. These conflicting results could be explained by an increased drive to eat in amino acid deprived flies perhaps interacting with the drive of the fly to discriminate.

3.4

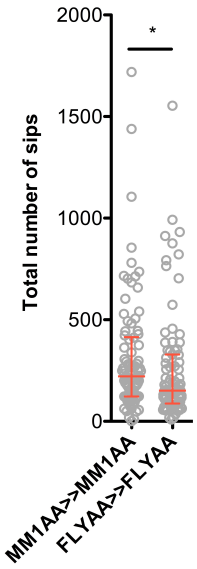


Figure 3.4. Flies feed slightly less on FLYAA than MM1AA. Total number of sips on yeast of Dahomey flies maintained on the food type indicated on the x-axis for 3 days. n=83-86 for all conditions; Mann-Whitney test; error bars represent the median and IQR; $p<0.05$*.

3.5

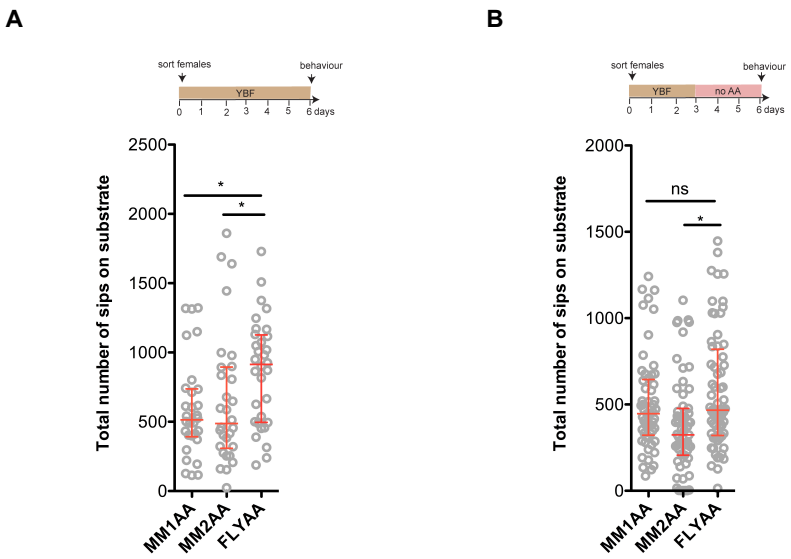


Figure 3.5. Exome-matched food is more appetitive depending on the internal state of the fly. (A and B) Total number of sips taken by Dahomey flies on the substrates that are labeled on x-axis, **(A)** after flies were prefed a diet of YBF or **(B)** no amino acids. n=30-64 for all conditions; outliers removed [(IQR1-(1.5*IQR) and IQR3+(1.5*IQR)]; Kruskal-Wallis test followed by Dunns multiple comparisons; error bars represent the median and IQR; p>0.05 ns, p<0.05*.

These results are in accordance with the amino acid ratio of FLYAA being more satiating – not only do the flies eat less of FLYAA, they do this even when they find the food source more appetitive than the non-matched foods.

3.5. Discussion

These results demonstrate that an exome-matched diet is more satiating for the fly, and that we can uncouple this characteristic from the putative confounding effects of a simple increase in food-intake or lack of appetitveness. This suggests that the animal has a requirement for a specific ratio of amino acids, and this ratio may impact the ability of the animal to utilise the amino acids efficiently in that diet. Indeed it was shown that the a diet in which one amino acid is limiting can inhibit the use of the others [6]. Interestingly, the flies not only do not consume more of the exome-matched diet, they show a tendency to eat less. This is consistent with the hypothesis that, if the right balance of amino acids is present, the nutrients can be used more efficiently for cellular processes, which in turn, removes the need for consumption of more food. Furthermore, the observation that these animals are more satiated, and at the same time consume less may account for the other differences that have been noted in flies between these diets. Flies fed FLYAA exhibited a higher rate of egg laying in early life, with no reduction in lifespan. Studies so far discuss this reproduction-lifespan trade-off in terms of amount of protein consumed, with high amounts being optimal for reproduction, but limiting for lifespan. It is possible that an exome matched diet, supplying all the amino acids in the

right proportions is sufficient to fuel optimal egg laying without causing any detrimental effects to the animal leading to reduced lifespan. This possibility was already implicated in an earlier publication where it was noted that flies fed a restricted diet (where all the nutrients are diluted), exhibited an increased lifespan at the expense of reproduction [6]. Adding a mix of only amino acids to this restricted diet increased reproduction but decreased lifespan. However, addition of only methionine to the restricted food uncoupled these two effects, suggesting the balances of amino acids is crucial in determining these life-history traits.

It is interesting that the exome-matched diet is apparently more appetitive that non-exome matched. The notion that animals reject imbalanced food sources is not new [7, 11], but seen here in the context of an exome-matched and unmatched diet strengthens this model. Furthermore, there is evidence that the rejection of imbalanced food goes though the general control nonderepressing 2 (GCN2) pathway [11], so the next question would be to determine if an exome-matched diet also impacts this signalling pathway, or perhaps other pathways in the animal.

This work contributed to a publication [1] in which our collaborators also observed that exome-matching improved growth, reproduction and lifespan in mouse. Together with my work, this data suggests that exome-matching does indeed represent a quantitative method for designing 'balanced' diets, by enhancing the biological efficiency of food, leading to improved health-span across phyla.

3.6. References

1. Piper, M. D. W. *et al.* Matching Dietary Amino Acid Balance to the In Silico- Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan. *Cell Metab.* **25,** 610–621 (2017).

2. Simpson, S. J. & Raubenheimer D. *The Nature of Nutrition*.

3. Lee, K. P. *et al.* Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proc. Natl. Acad. Sci.* **105,** 2498–2503 (2008).

4. Raubenheimer, D., Machovsky-Capuska, G. E., Chapman, C. A. & Rothman, J. M. Geometry of nutrition in field studies: an illustration using wild primates. *Oecologia* **177,** 223–234 (2015).

5. Simpson, S. J. & Raubenheimer, D. Obesity: the protein leverage hypothesis. *Obes. Rev.* **6,** 133–142 (2005).

6. Grandison, R. C., Piper, M. D. W. & Partridge, L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* 1–5 (2009).

7. Hao, S. *et al.* Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307,** 1776–8 (2005).

8. Leib, D. E. & Knight, Z. A. Rapid Sensing of Dietary Amino Acid Deficiency Does Not Require GCN2. *Cell Rep.* **16,** 2051–2052 (2016).

9. Piper, M. D. W. *et al.* A holidic medium for Drosophila melanogaster. *Nat. Methods* **11,** 100–5 (2014).

10. Lee, W. C. & Micchelli, C. A. Development and Characterization of a Chemically Defined Food for Drosophila. *PLoS One* **8,** 1–10 (2013).

11. Bjordal, M. *et al.* Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in Drosophila. *Cell* **156,** 510–21 (2013).

12. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and

neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

13. Itskov, P. M. *et al.* Automated monitoring and quantitative analysis of feeding behavior in Drosophila. *Nat. Commun.* 4560 (2014).

14. Hunt, V. A qualitatively minimal amino acid diet for D. melanogaster. *Dros. Inf. Serv.* 179 (1970).

15. Leitão-Gonçalves, R. *et al.* Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLOS Biol.* **15,** e2000862 (2017).

3.7. Acknowledgements

Dr Matthew Piper, the lead author on the publication this work contributed to, for collaboration and sharing the unpublished diets.

Dr Pavel Itskov for help with MatLab processing of FlyPAD data.

Chapter 4. Cellular responses in neurons correlated with food choice behaviour

4.1. Summary

If an animal is deprived of an important nutrient it will search out and eat food to replenish this missing nutrient. This is known as homeostatic feeding behaviour. Neuronal mechanistic Target Of Rapamycin (mTOR) signalling has been implicated as necessary for this behaviour, however, the exact nature of the cellular and molecular changes that take place in the neurons in response to changing dietary conditions remains unknown. In this chapter, I describe the results that show both mTOR signalling and autophagic activity respond in fly heads to amino acids. Furthermore, I use an arginine sensor and find that dietary manipulations of amino acids induce fluctuations in the neuronal levels of this essential amino acid. These results support a role for both neuronal mTOR pathway/autophagy and free amino acid availability in mediating neuronal responses to nutrient stress, and implicate both pathways in the modulation of homeostatic feeding.

4.2. Introduction

In the previous chapter I discussed the importance of a precise ratio of nutrients for a balanced diet, namely the protein:carbohydrate ratio, and the ratio between different amino acids. Another important aspect of a balanced diet is whether or not the macro- or micronutrient is actually present. It has been shown that feeding flies for a period of time with a protein-

deficient diet, or a diet lacking essential amino acids, will modify their feeding behaviour – quantifiable as changes in yeast preference in a 2-choice colour assay [1, 2]. This is termed homeostatic food choice, as the animal modifies the food it eats to return to a particular set-point. But how is this homeostatic feeding behaviour achieved at the molecular level and what molecular pathways respond in neurons to drive the changes seen in feeding behaviour?

The mTOR kinase is a highly conserved serine/threonine kinase that acts in a pathway of cascading phosphorylations [3]. The mTOR pathway responds to a variety of environmental cues, including changes in amino acid availability, as well as hormonal signals **(Figure 2.1)**. In rodents, hypothalamic mTOR was shown to regulate bulk food intake [4]. In *Drosophila*, neuronal mTOR signalling has been proposed to be required for the modulation of homeostatic feeding behaviour [1, 5]. As such, the pathway represents an ideal entry point for determining the molecular players of homeostatic feeding behaviour.

Numerous cellular processes lie downstream of the mTOR pathway, and may represent mechanisms by which alterations in mTOR signalling can modify neuronal activity **(Figure 2.1)**. One of these downstream pathways, autophagy, was originally identified as a response to starvation in mammalian cells [6]. Autophagy is now known to fulfil a variety of roles related to cellular homeostasis in higher eukaryotes [7]. In the nervous system autophagy has been shown to be necessary in the hypothalamus for food intake following a period of starvation in mice [8]. The detailed mechanisms of autophagy are known mostly from work in yeast and cell culture. Upon induction of

autophagy, vesicles grow to engulf parts of the cytoplasm and fuse with lysosomes. Following this fusion event the contents of the membrane bound vesicle are degraded and duly recycled by the cell **(Figure 2.2)** [9].

In this chapter I describe neuronal cellular and molecular responses to diets that lack either protein or specific amino acids, and discuss the implications for the correlation of these cellular and molecular changes with alterations in food choice behaviour. I developed and optimised methods to study how manipulations of dietary proteins or amino acids change the activity of either neuronal mTOR signalling or autophagy. Furthermore, I looked for evidence of amino acid fluctuations in neurons using an arginine sensor.

4.3. Materials and Methods

Fly rearing, media and dietary treatments: w^{1118} flies were reared in yeast-based food containing (per litre of water: 80g sugar cane molasses, 22g sugarbeet syrup, 8g agar, 80g corn flour, 10g soya flour, 18g yeast extract, 8ml propionic acid, 12 ml nipagin (15% in EtOH)). Upon emerging as adults, groups of 3-6 days old flies (15 females and 5 males) were transferred to fresh yeast based food for 3 days and then to fresh food for another 3 days, either yeast based food, 100mM sucrose, full holidic medium or holidic medium without amino acids. Where holidic media was used, preparation was according to Piper et al., 2014 and 2017 [10, 11].

Fly stocks: w^{1118}. w^{1118}; *nSyb-Gal42.1* (Vienna Drosophila Resource Center). w^{1118}; *UAS-GFP::Atg8a* (gift from TP Neufeld).

w^{1118}; elav-Gal4. *Atg8a[GD43096]* RNAi lines (Vienna Drosophila Resource Center).

Protein extraction, SDS-PAGE and Western analysis

P-Thr398 dS6K blots: 3-6 days aged female fly heads (60) were collected and immediately frozen on dry ice. They were homogenized in 150 µl of 1x laemmli solution with protease and phosphatase inhibitors (Pierce), and then heated to 95°C for 5 minutes. The homogenates were centrifuged at 4000 rpm for 2 minutes. 25 ul per sample was loaded and resolved on a 12% gel, followed by electroblotting to nitrocellulose membranes (Li-cor). Subsequently, blots were probed with a polyclonal anti-P-Thr398 dS6K antibody (1:500, CST 9209S), a polyclonal anti-Histone H3 antibody (1:2500, CST), anti-dS6K antibody (1:5000, gift from A. Telemann) and a monoclonal anti-actin antibody (1:2000, Sigma). Immunoblots were scanned using an Amersham Imager 600. Intensity analysis was performed using the Fiji software (using the gels and measurement functions). Relative amounts of the P-Thr 398 dS6K, and total dS6K protein of individual samples were calculated and corrected using the histone H3 protein as loading control. Statistical analysis was done in Microsoft Excel and GraphPad Prism software using a t-test.

Atg8a blots: Fly heads (60) were collected from 3-6 days aged frozen females by vortexing. Heads were homogenized in 80 µl of solubilisation buffer (2% SDS with protease inhibitors, Pierce). The homogenates were centrifuged at 13,000 rpm for 5 minutes, and the supernatant removed, and diluted in 2x laemmli solution. The samples were heated at 95°C for 5 minutes. 25 ul per sample was loaded and resolved on a 15% gel, followed by

electroblotting to nitrocellulose membranes (Li-cor). Subsequently, blots were probed with a polyclonal anti-GABARAP antibody (1:1000, MBL), a polyclonal anti-Histone H3 antibody (1:2500, CST) and a monoclonal anti-actin antibody (1:2000, Sigma). Immunoblots were scanned using an Amersham Imager 600. Intensity analysis was performed using Fiji software (using the gels and measurement functions). Relative amounts of Atg8a of individual samples were calculated and corrected using the histone H3 protein as loading control. Statistical analysis was done in Microsoft Excel and GraphPad Prism software using a one-sample t-test.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from female heads (approx. 50) with Purezol (Biorad). RNA samples were then reverse-transcribed using iScript Reverse Transcription Supermix (Biorad) according to manufacturer's instructions. Quantitative real-time PCR was carried out using SsoFast EvaGreen Supermix (Biorad) and a CFX96 Real Time System (Biorad) according to manufacturer's instructions. Relative mRNA levels were calculated using the qbase+ software. *Rpl32* and *actin42A* were used as reference genes. Below is the list of primers used in this study and corresponding DNA sequence:

unk_fwd - 5' GCCCATGTGGAACCTTGC 3'
unk_rvs - 5' GCGCCGAGGAACGTGTTA 3'
ref(2)P_fwd - 5' GTCTCCTGAAACGGGCAAT 3'
ref(2)P_rvs - 5' TGGATCGACGCTGATAAAGA 3'
Actin42A_fwd - 5' CAGGCGGTGCTTTCTCTCTA 3'
Actin42A_rvs - 5' AGCTGTAACCGCGCTCAGTA 3'

RpL32_fwd - 5' GCCCAAGATCGTGAAGAAGC 3'
RpL32_rvs - 5' GCACTCTGTTGTCGATACCCTTG 3'

Immunostaining and Imaging

Female or male flies were dissected in PBS containing 4% PFA, and fixed in PBS containing 4% PFA for a further 20-30 minutes. After fixation, the brains were washed twice in PBST, and once in PBS before mounting in Vectashield (VectorLaboratories). The brains were imaged under a confocal laser scanning microscope LSM 710 (Zeiss). Image processing and analysis were done with Fiji (National Institutes of Health) and Photoshop CS5 (Adobe). Average fluorescence projections were compiled for each brain, background was subtracted and the average fluorescence/unit area was calculated. For the experiments normalised to mCherry, the average fluorescence/unit area from the GFP channel was subtracted from the average fluorescence/unit area from the mCherry channel.

4.4. Results

4.4.1. The mTOR pathway reacts in the fly head to dietary amino acids

mTOR activity can be monitored in cells by quantification of changes in multiple downstream effector pathways, such as transcription and translation. For example, mTOR is known to directly phosphorylate S6 kinase (S6K) at threonine 398, and this phosphorylation has been widely used as a readout for mTOR activity in both mammals and *Drosophila* [12, 13]. The inhibition of mTOR by amino acid removal or addition of rapamycin has been

shown to decrease the phosphorylation of the Thr398 in S6K [12]. In addition, *unk/CG4620* transcript levels (a gene with roles in mRNA binding and ubiquitination) responds to mTOR activity, and the transcriptional upregulation of this gene has been shown to be part of a cellular metabolic response to nutrient stress [14, 15]. Using both the phosphorylation of dS6K and transcriptional regulation of *unk* as reporters for change in neuronal mTOR signalling, I asked if mTOR activity was modified in flies subjected to diets with or without protein or amino acids.

In order to detect changes in these mTOR reporters as a response to protein availability, I used head protein extracts prepared from w^{1118} flies kept for three days on yeast based food or sucrose. I observed that the removal of protein from the fly's diet, by pre-feeding the fly sucrose only, results in a decrease in the mTOR-dependent phosphorylation of dS6K at Thr398 when compared to flies that had been kept on a diet containing protein **(Figure 4.1A)**. This decrease in P-Thr398 dS6K was significant when compared to the total amount of dS6K **(Figure 4.1B)**, or only to the loading control **(Figure 4.1C)**. Moreover, the removal of amino acids (AAs) from the fly's diet was sufficient to induce these changes **(Figure 4.1D)**. I used head protein extracts prepared from w^{1118} flies kept for three days on diets with or without all AAs. I observed that the removal of AAs from the fly's diet resulted in a decrease in the mTOR-dependent phosphorylation of dS6K at Thr398 when compared to flies that had been kept on a diet containing all AAs. This decrease in P-Thr398 dS6K was significant when compared to the total amount of dS6K **(Figure 4.1E)**, or only to the loading control **(Figure**

4.1

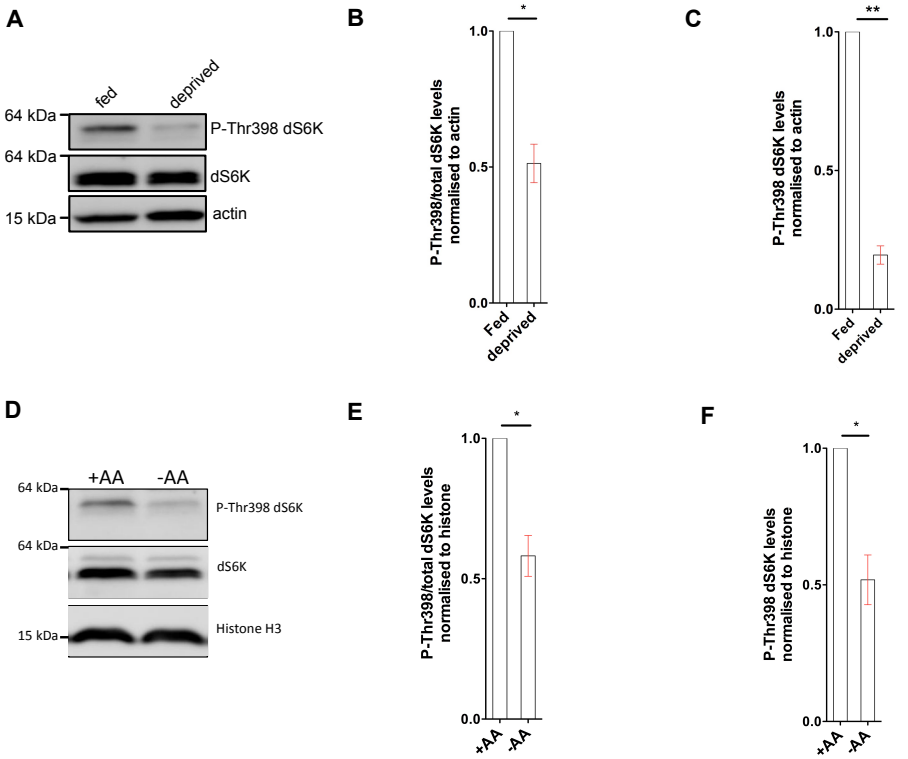


Figure 4.1. Absence of protein or amino acids in the diet decreases mTOR signalling in *Drosophila* heads. **(A-F)** mTOR activity was measured by immunoblot with anti-phospho-T398 dS6K (p-dS6K) antibody and anti-dS6K antibody. Actin or Histone H3 was used as the loading control as indicated. Head protein extracts were prepared from w^{1118} flies prefed yeast based food (fed), 100mM sucrose (deprived), holidic media with amino acids (+AA) or without amino acids (-AA). **(B and E)** Levels of P-dS6K as a ratio of total dS6K, normalised to the loading control indicated. **(C and F)** Levels of P-dS6K normalised to the loading control indicated. Data shown as mean, error bars are the standard error of the mean. Significance tested using t-test; $p<0.05$*, $p<0.01$**. **(A-C)** n=2. **(D-F)** n=4.

4.2

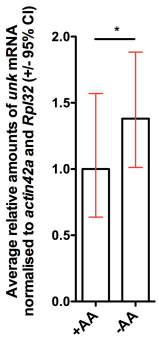


Figure 4.2. Absence of amino acids in the diet decreases mTOR signalling in *Drosophila* heads. *unk* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. RNA head extracts were prepared from female flies prefed holidic media with amino acids (+AA) or without amino acids (-AA). n=3; significance tested using t-test; data shown as mean, error bars show 95% CI; p<0.01**.

4.1F). Furthermore, I compared the levels of *unk* mRNA in head RNA samples prepared from flies kept on food with or without dietary AAs. I found that *unk* transcript is significantly higher in the heads of flies kept on an amino acid deficient diet **(Figure 4.2)**.

Together these results indicate that reducing the flies access to dietary protein or amino acids is sufficient to reduce mTOR pathway activity in the heads of flies.

4.4.2. Neuronal autophagy responds to dietary amino acids

The activation of autophagy can be divided into multiple quantifiable processes, which can be used to assay the level of activity in the pathway. Notably, the activation of the pathway induces the formation of membrane bound vesicles via the recruitment of Autophagy related protein 8a (Atg8a) from the cytoplasm to the growing double membrane structure that will close to form the autophagosome **(Figure 4.3A)**. Relative levels of cytoplasmic Atg8a and membrane bound Atg8a, can therefore be used as a measure of autophagy activity. In addition, the autophagic vesicles grow to engulf parts of the cytoplasm, organelles and sometimes specific cargo proteins, such as p62/ref(2)P. The degradation of the cargo protein p62/ref(2)P and the upregulation of the *p62/ref(2)P* transcript can also be used to quantify autophagy activity [16]. I assessed the levels of autophagy in the heads of flies in response to changes in the

availability of dietary protein or amino acids by quantifying changes in both Atg8a localisation and *ref(2)P* transcript levels.

Atg8a is expressed in the head of w^{1118} flies in two protein forms, running at different molecular weights in an electrophoresis assay: the higher molecular weight cytoplasmic form, Atg8a I, and the lower molecular weight membrane bound form, Atg8a II **(Figure 4.3B)** [16]. I found that in the heads of protein deprived w^{1118} flies there is a trend to increase in the ratio of membrane bound to cytoplasmic Atg8a protein **(Figure 4.3B and C)**, indicating that autophagic activity has been stimulated by this dietary manipulation. The removal of only amino acids from the fly's diet also results in a trend of an increase in the ratio of membrane bound to cytoplasmic Atg8a protein **(Figure 4.3 D and E)**. These results support the hypothesis that amino acids in dietary protein drive autophagic changes in the heads of flies. Importantly, the signal measured in head extracts should correspond mostly to the protein expressed in the nervous system, and not in other head tissues, such as fat body, since RNAi mediated knockdown of *Atg8a* in the nervous system alone drastically reduces the levels of Atg8a seen by immunoblot **(Figure 4.3F)**. Indicating that mTOR activity can indeed drive similar changes to protein deprivation in autophagy.

A different way to analyse the localisation of Atg8a is to make use of a transgenic fly line expressing a GFP bound form of Atg8a. GFP::Atg8a recapitulates the behaviour of Atg8a upon autophagy activation: it is recruited from the cytoplasm to the growing autophagosomes and binds both the outer and inner membrane of the autophagosome. Fusion of the autophagosome

4.3

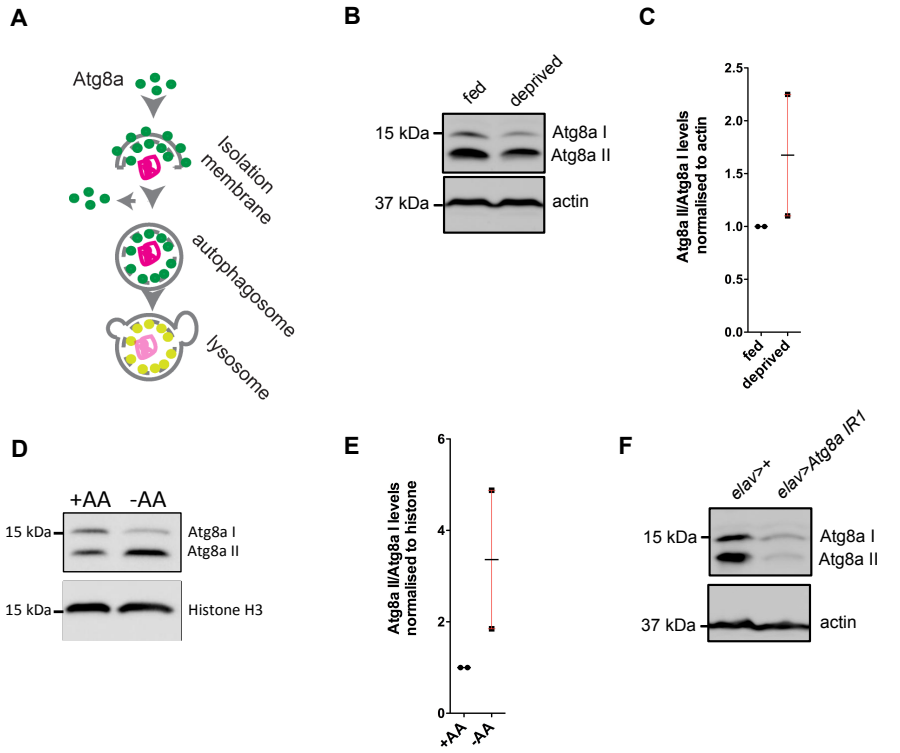


Figure 4.3. Absence of protein or amino acids in the diet promotes autophagy in *Drosophila* heads. (A) A diagram illustrating Atg8a recruitment to growing autophagosomes followed by degradation after the vesicle fuses with the lysosome. Atg8a is shown as green circles, cellular contents to degrade in pink, degraded Atg8a in yellow and degraded cellular contents in light pink. **(B-F)** Autophagic activity was measured by immunoblot with anti-GABARAP/Atg8a antibody. Head protein extracts were prepared from w^{1118} flies prefed yeast based food (fed), 100mM sucrose (deprived), holidic media with amino acids (+AA) or without amino acids (-AA). Actin or Histone H3 was used as the loading control. n=2. **(C and E)** The ratio of membrane bound Atg8a (Atg8a II)/cytoplasmic Atg8a (Atg8a I) normalised to the loading control indicated. Data shown as mean and error bars the standard error of the mean. **(F)** Autophagic activity measured by immunoblot with anti-GABARAP/Atg8a antibody in head protein extracts from flies where Atg8a was pan-neuronally knocked down. Actin was used as the loading control. n=2.

4.4

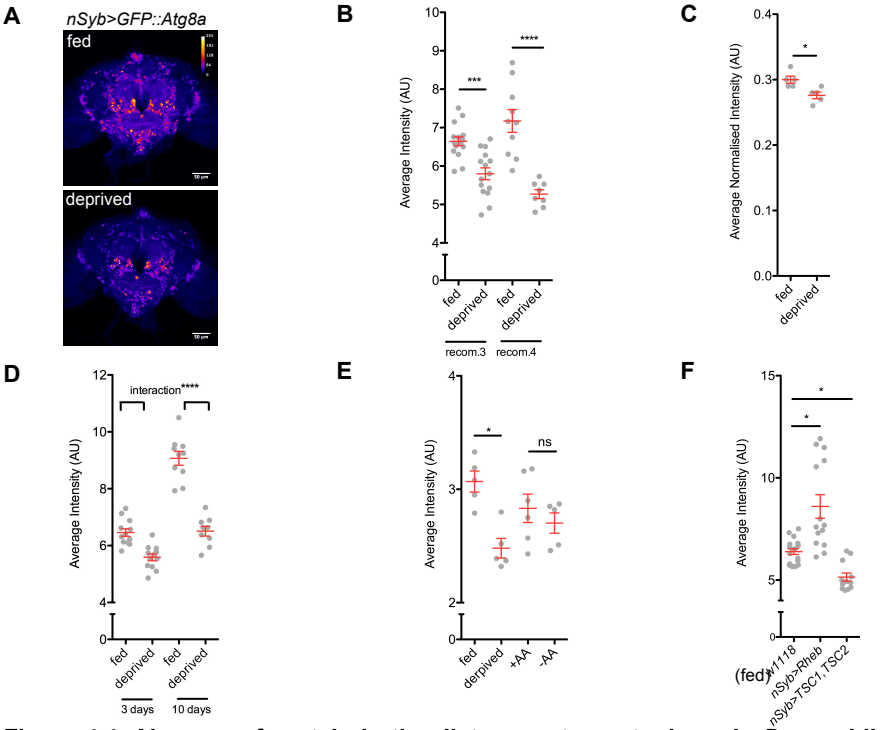


Figure 4.4. Absence of protein in the diet promotes autophagy in *Drosophila* brains. **(A-F)** Flies were prefed one of the following diets: yeast based food (fed), 100mM sucrose (deprived), holidic media with amino acids (+AA), holidic media without amino acids (-AA), as indicated in the graphs. **(A)** Average fluorescence projections of female brains dissected from flies prefed the indicated diets. Hot colours indicate areas of higher signal intensity, and cold colours indicate areas of lower signal intensity. Scale bars 50µm. **(B-F)** The average fluorescence/unit area was calculated from the average fluorescence projections of brains dissected from flies of various genotypes and dietary manipulations. **(B)** Transgenic flies expressing *nSybGal4* recombined with UAS-*GFP::Atg8a* (2 lines are shown, recom. 3 and recom.4), **(C)** *nSyb>GFP::Atg8a (recomb. 3); mCherry*, **(D)** *nSyb*>UAS-*GFP::Atg8a (recom.3)* males, **(E)** *nSyb*>UAS-*GFP::Atg8a* (recom.3) females, **(F)** genotypes indicated on x-axis. **(B-F)** n=4-10. Data shown as mean and standard error of the mean; (B, C and E) significance tested for using t-test, (F) Kruskal-Wallis test followed by Dunns multiple comparisons or (D) 2-WAY anova ; p>0.05 ns, p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

with lysosomes results in the quenching and degradation of the GFP::Atg8a on the inner membrane by the increased acidic conditions and lysosome enzymes [17]. By measuring the change in fluorescence intensity of GFP::Atg8a I can follow the dynamics of the activity of the pathway. To monitor Atg8a dynamics in the nervous system of the fly, I induced the expression of GFP::Atg8a in the entire nervous system of the fly using the pan-neuronal Gal4 driver, *nSyb-Gal4*. I then quantified changes in the localisation of Atg8a by measuring the fluorescence intensity of GFP::Atg8a. Three days of protein deprivation (100mM sucrose) results in a decrease in neuronal GFP::Atg8a, when comparing the average fluorescent intensity to flies fed a diet containing protein **(Figure 4.4A and B)**. Importantly, the decrease in the GFP::Atg8a is still significant after normalisation to the unspecific translation control mCherry **(Figure 4.4C)**. Indicating that the decreased GFP::Atg8a signal is not a result of general transcriptional/translational changes. The decrease in GFP::Atg8a is indicative of its increased quenching/degradation following lysosomal fusion with the autophagosome, and suggests neuronal autophagic activity is increased in the protein-deprived flies.

I next asked if I could use GFP::Atg8a localisation as a reporter for increasing levels of protein appetite. Whilst females increase their preference for yeast strongly after three days of protein deprivation, males take up to ten days to exhibit the same behaviour [1]. I compared GFP::Atg8a in brains taken from males after three and ten days of protein deprivation. Both 3 and 10 days of deprivation induced significant decreases in the GFP signal of Atg8a. However, ten days of protein deprivation

induces a larger decrease in the GFP signal in male brains when compared to fully fed males (interaction $p<0.0001$), compared to the decrease in signal seen after only three days of protein deprivation **(Figure 4.4D)**. These results show that GFP::Atg8a localisation correlates well with protein appetite.

I next asked if amino acid deprivation could recapitulate the effects seen in GFP::Atg8a following protein deprivation. After three days of being fed a holidic diet with no amino acids (-AA) the brains of female flies failed to show any significant decrease in GFP::Atg8 **(Figure 4.4E)**. The lack of effect seen here stands in contrast to the other data suggesting neuronal autophagy can indeed be induced by lack of dietary amino acids. Testing a longer period of amino acid deprivation may help to solve this question.

As previously mentioned, mTOR activity negatively regulates autophagic activity. In order to ask if this assay was not only sensitive to dietary manipulations, but also to mTOR manipulations, I induced the overexpression of the known mTOR inhibitors, TSC1 and TSC2, and an mTOR activator, Rheb, in the nervous system of flies also overexpressing GFP::Atg8a. Brains of fully fed mated females overexpressing Rheb show a significant increase in GFP::Atg8a when compared with control flies **(Figure 4.4F)**. Furthermore, brains from flies overexpressing TSC1 and TSC2 show a significant decrease in GFP::Atg8a when compared with control females **(Figure 4.4F)**.

Ref(2)P is yet another marker of autophagic activity, with increased autophagic activity correlated with higher transcript

levels of this gene [16]. Quantification of the *ref(2)P* transcript levels in head extracts of flies previously fed a holidic diet with (+AA) and without (-AA) amino acids, shows that transcription of this gene is increased in the absence of amino acids **(Figure 4.5**).

Taken together, the results obtained with both the Atg8a and ref(2)P assays indicate that the levels of autophagic activity in the heads of flies increase with low dietary protein levels, and mirror the changes in feeding behaviour exceptionally well. Furthermore, it appears to be specifically the changes in amino acid levels driving these changes. These changes in autophagic activity also respond to genetic manipulations of the mTOR pathway. Finally, these results are consistent with my data indicating that both protein and amino acid deprivation can lead to a decrease in mTOR activity in the central nervous system.

4.4.3. Testing an arginine sensor

The correlation of neuronal mTOR and autophagy activity with dietary protein conditions suggests that dietary amino acids could be changing amino acid levels in the brain, which in turn drive changes in mTOR and autophagy. In order to answer the question of whether neuronal amino acids could be driving changes in neuronal mTOR and autophagy I established a method to assess changes in a specific amino acid, arginine, directly in neurons.

In collaboration with Loren Looger at Janelia Farm, flies with a genetically encoded arginine sensor were generated. The

4.5

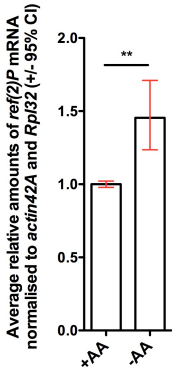


Figure 4.5. Absence of amino acids in the diet promotes autophagy in *Drosophila* heads. *ref(2)P* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. RNA head extracts were prepared from female flies prefed holidic media with amino acids (+AA) or without amino acids (-AA). n=3; significance tested using t-test; data shown as mean, error bars show 95% CI; $p<0.01$**.

sensor is composed of one part that is the amino acid binding domain of a bacterial Solute Carrier 7, SLC7/CAT amino acid transporter that is mutated to only bind arginine. The other part is a permutated GFP which fluoresces only when free arginine is bound to the SLC7/CAT transporter domain **(Figure 4.6A)**. This sensor has no subcellular targeting sequence, and localises to the cytoplasm of the cell, hence its name SLC7/CAT-cyto. The binding specificity of the mutated transporter was previously confirmed by our collaborators using a cell-free system (data not shown). Arginine is an essential amino acid, meaning it cannot be synthesised by the fly, and so it should be representative of neuronal amino acid changes following removal of all protein or amino acids from the diet. Importantly, removal of arginine from the flies diet is sufficient to induce an increase in yeast preference in a 2-colour food choice assay [2].

I used *nSyb-Gal4* to drive expression of this sensor pan-neuronally in protein fed female flies. I found that SLC7/CAT-cyto expression is strong and non-uniformly distributed across different cell populations in the brain, with apparently stronger expression in the pars intercerebralis and the antennal lobes **(Figure 4.6B and C)**. I compared fluorescent signal measured in brains taken from flies kept on food with (fed) or without protein (deprived). The GFP signal intensity was lower in the brains dissected from flies previously fed a diet lacking protein **(Figure 4.6D)**. A second version of the sensor was designed, targeting it to cell membranes, SLC7/CAT-memb, where the amino acid binding domain is extracellular/luminal. The intensity of fluorescence resulting from the membrane bound form of the sensor increases following protein deprivation **(Figure 4.6E)**.

4.6

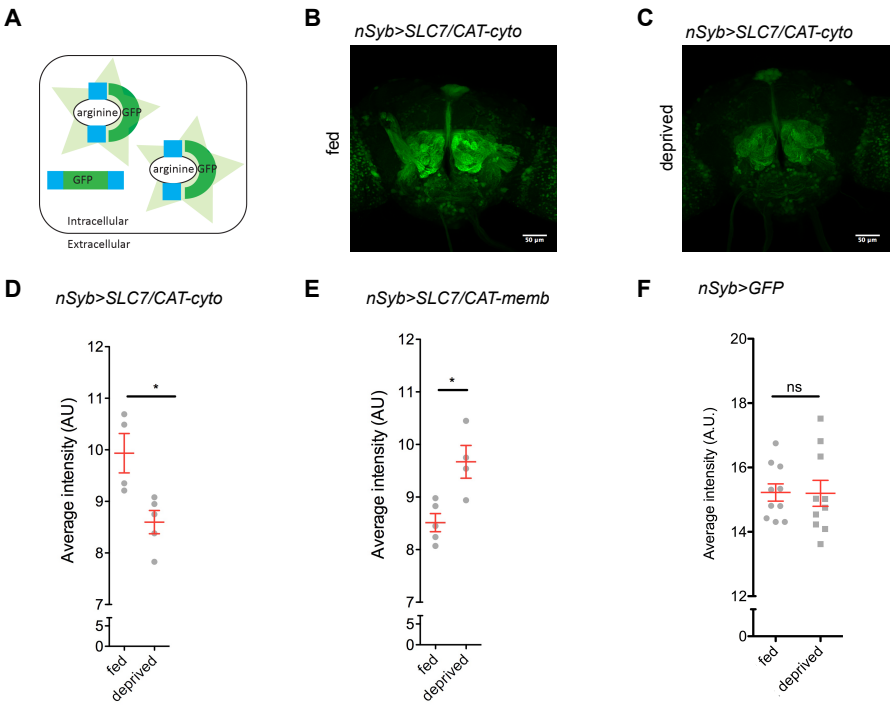


Figure 4.6. The arginine sensor SLC7/CAT responds in the nervous system to protein deprivation (A) Diagram of SLC7/CAT-cyto. Permutated GFP is a dark green arch, the SLC7/CAT-cyto is split in two, represented by two blue squares, when arginine binds, the sensor fluoresces. **(B-D)** Flies were prefed either yeast based food (fed) or 100mM sucrose (deprived). **(B and C)** Average fluorescence projections of *nSyb>SLC7/CAT-cyto* brains dissected from females prefed the indicated diets. **(D-F)** The average fluorescence/unit area was calculated from the average fluorescence projections of brains dissected from flies of various genotypes and dietary manipulations. **(D)** Average intensity of SLC7/CAT-cyto expressing brains dissected from flies prefed with indicated diets. n=4-6. **(E)** Average intensity of SLC7/CAT-memb expressing brains dissected from flies prefed with indicated diets. n=4-6. **(F)** Average intensity of brains dissected from *nSyb>GFP* flies, prefed indicated diets. n=10. **(D-E)** Data shown as mean and standard error of the mean. Significance tested using t-test. $p>0.05$ ns, $p<0.05$*.

4.7

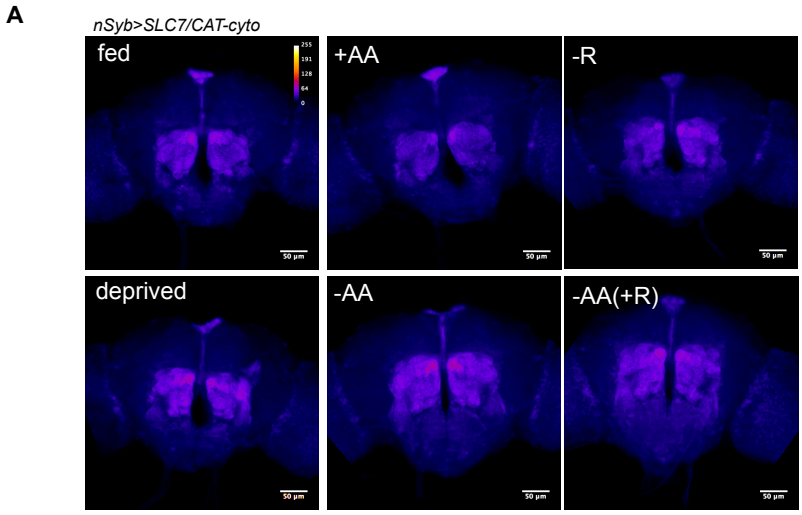


Figure 4.7. The arginine sensor responds in the nervous system to amino acid deprivation. *nSyb>SLC7/CAT-cyto* expressing female flies were prefed one of the following diets: yeast based food (fed), 100mM sucrose (deprived), holidic media with amino acids (+AA), holidic media without amino acids (-AA), holidic media without arginine (-R), holidic media without all amino acids apart from arginine (-AA(+R)). **(A)** Average fluorescence projections of brains dissected from flies prefed with indicated diets. Scale bar 50 µm. Hot colours indicate high signal, and cold colours low signal. **(B)** Average intensity values of imaged brains dissected from flies prefed indicated diets. n=10. Data shown as mean and standard error of the mean. Significance tested using t-test. $p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****.

The differences in fluorescence intensity seen between SLC7/CAT-cyto and SLC7/CAT-memb could be indicative of the complex dynamics of amino acids inside and outside of cells. One hypothesis is that free arginine might decrease in the cytoplasm following a reduction in availability of dietary protein because it is being used up, whilst, inside membrane bound subcellular compartments, such as lysosomes, the level of free arginine increases, perhaps as a result of processes such as autophagy that feed proteins into these subcellular compartments. In addition, extracellular free arginine may increase as a result of protein reserves being released from elsewhere in the fly into circulating haemolymph or extracellular environment. Notably, there are no changes in signal intensity between brains dissected from fed and deprived flies when only cytoplasmic GFP was pan-neuronally overexpressed **(Figure 4.6F)**.

To determine if removal of amino acids from the fly's diet phenocopies protein removal from the diet in terms of changes in fluorescence intensity of SLC7/CAT-cyto I quantified the GFP signal intensity of brains dissected from flies that had been fed diets with or without amino acids. I again expressed the SLC7/CAT-cyto pan-neuronally in flies that had been kept on food with or without amino acid for three days. Amino acid deprived females exhibited an increased GFP signal intensity in brains compared to the flies that had been fed a diet containing amino acids **(Figure 4.7A and B)**. These results suggest that after feeding a diet where only amino acids have been removed, there is a response in neurons that leads to increased levels of free arginine. This also suggests that dietary protein deprivation

and dietary amino acid deprivation do not elicit the same responses in arginine levels in neurons.

I next asked if the specific removal of arginine from the fly's diet is sufficient to induce an increase in free arginine in neurons. Prefeeding the flies a diet with all amino acids apart from arginine (-R) resulted in an increase in GFP signal compared to amino acid fed flies. To determine if arginine alone was sufficient to induce this change in fluorescence intensity I deprived flies of all amino acids apart from arginine (-AA+R). If lack of dietary arginine alone was driving changes in the fluorescence intensity, the fluorescence in the brains dissected from flies deprived of all amino acids apart from arginine (-AA+R) should not differ from the fluorescence intensity of the brains of flies fed a diet containing all amino acids. This was not the case, brains dissected from flies prefed on -AA+R also showed an increase in fluorescence intensity when compared to amino acid fed flies. These results indicate that there is a mechanism in neurons that is sensitive to the removal of one amino acid from the fly's diet, arginine, but appears not to be dependent on the removal of that specific amino acid, as both the removal of arginine alone or all amino acids apart form arginine induce this response. This mechanism is sensitive to amino acids, and leads to increased free arginine levels.

4.5. Discussion

The results presented in this chapter support a hypothesis that dietary protein or more specifically, dietary amino acids, seem to

modulate mTOR pathway signalling. I show evidence for the modulation of the mTOR pathway by dietary protein and amino acids, as quantified by the phosphorylation of dS6K. I also present data showing how the pathways downstream of mTOR are changed by dietary protein/amino acid availability, seen by the changes in *unk* transcript, and autophagy activity. Changes in the levels of *unk* transcript have been characterised as part of a nutrient homeostasis response in the cell. The elevation of autophagy is associated with the degradation of a whole plethora of proteins and organelles within the neuron, and the recycling of amino acids from proteins.

There still remains some doubt as to the neuronal specificity of these cellular responses. Head extracts contain fat body tissue, trachea, hemocytes and cuticle as well as neuronal and glial tissue. I have attempted to address this problem in two ways: I have shown biochemically that most of the endogenous Atg8a signal comes from neurons; and I also quantified GFP::Atg8a changes via fluorescence intensity when expressing the fusion protein specifically using a neuronal driver. In future work, it will be important to optimise these biochemical assays with brain extracts, and to make use of tagged proteins that can be expressed only in the nervous system to quantify changes in the mTOR pathway, to remove any doubt as to the origin of mTOR pathway changes.

The pan-neuronal expression of the arginine sensor, SLC7/CAT-cyto and SLC7/CAT-memb, seems to allow the visualisation of fluctuations of free arginine in neurons. The experiments done using this sensor indicate that manipulations of dietary amino acids appear to have strong effects on levels of free arginine in

neurons. Furthermore, the results hint at underlying mechanisms for the control of amino acid homeostasis inside neurons. However, SLC7/CAT-cyo and -memb are still very preliminary tools. Although the binding specificity of this sensor was tested in cell-free systems, this has not been repeated in a cellular context, and this is a vital experiment to do before further research is done with this tool. After all, the cytoplasm of a cell and an in vitro setup are very different in terms of concentrations of molecules present that could interfere or compete with the binding of the arginine sensor to arginine.

Whilst feeding flies only sucrose to deprive them of protein is seen as a naturalistic dietary manipulation, the use of holidic media has allowed us to ask the question of whether amino acid deprivation alone is sufficient to drive the changes seen following protein deprivation. For both mTOR and autophagy activity, amino acid removal phenocopied a diet lacking protein. However, when looking at the levels of neuronal free arginine I noticed some striking differences. Whilst these experiments with the arginine sensor are very preliminary, and the tool requires further optimisation, the data gathered until now still warrants some consideration. The removal of protein from the fly's diet seems to reduce levels of free arginine, whilst the removal of amino acids from the fly's diet seems to increase the levels of free arginine. Other experiments done in the lab corroborate these results. Amino acid measurement data and metabolomics data collected from the heads of flies indicate levels of arginine decrease following protein deprivation, and increase following amino acid deprivation (data not shown). In the future, it will be important to determine if this difference disappears if flies are

deprived of amino acids for perhaps longer than three days. Furthermore, if amino acid deprivation increases cytoplasmic arginine, a known, potent activator of mTOR activity, why do I see evidence of decreased mTOR activity following the same dietary manipulation? Perhaps not all amino acids are equal, and it is fluctuations in others that drive the changes in mTOR activity? Or fluctuations in and around subcellular compartments, such as lysosomes, and not in the cytoplasm or cell membranes, need to be monitored in order to form correlations with mTOR activity.

Given my results that neuronal mTOR signalling as well as levels of free arginine, change depending on a fly's diet, there are two questions that arise: Could these changes in free amino acids directly drive mTOR and autophagy pathway activity in the heads of flies? Is the activity in the mTOR and autophagy pathways purely reactive to dietary changes, or are the pathways responding in a way that can drive the behavioural changes we see in food choice in response to these dietary manipulations?

4.6. References

1. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

2. Leitão-Gonçalves, R. *et al.* Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLOS Biol.* **15,** e2000862 (2017).

3. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124,** 471–484 (2006).

4. Cota, D. Hypothalamic mTOR Signaling Regulates Food Intake. *Sci. (New York, NY)* **312,** 927–930 (2006).

5. Vargas, M. A., Luo, N., Yamaguchi, A. & Kapahi, P. A Role for S6 Kinase and Serotonin in Postmating Dietary Switch and Balance of Nutrients in D. melanogaster. *Curr. Biol.* **20,** 1006–1011 (2010).

6. Schworer, C. & Mortimore, G. Glucagon-induced autophagy and proteolysis in rat liver: mediation by selective deprivation of intracellular amino acids. *Proc. Natl. Acad. Sci. USA* **76,** 3169–3173 (1979).

7. Yang, Z. & Klionsky, D. J. Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* **12,** 814–822 (2010).

8. Kaushik, S. *et al.* Autophagy in Hypothalamic AgRP Neurons Regulates Food Intake and Energy Balance. *Cell Metab.* **14,** 173–183 (2011).

9. Xie, Z. & Klionsky, D. J. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* **9,** 1102–1109 (2007).

10. Piper, M. D. W. *et al.* A holidic medium for Drosophila melanogaster. *Nat. Methods* **11,** 100–5 (2014).

11. Piper, M. D. W. *et al.* Matching Dietary Amino Acid Balance to the In Silico- Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan. *Cell Metab.* **25,** 610–621 (2017).

12. Hara, K. *et al.* Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* **273,** 14484–94 (1998).

13. Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T. P. & Guan, K.-L. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat. Cell Biol.* **10,** 935–945 (2009).

14. Guertin, D. A., Guntur, K. V. P., Bell, G. W., Thoreen, C. C. & Sabatini, D. M. Functional Genomics Identifies TOR-Regulated Genes that Control Growth and Division. *Curr. Biol.* **16,** 958–970 (2006).

15. Tiebe, M. *et al.* Article REPTOR and REPTOR-BP

Regulate Organismal Metabolism and Transcription Downstream of REPTOR and REPTOR-BP Regulate Organismal Metabolism and Transcription Downstream of TORC1. *Dev. Cell* 272–284 (2015).

16. Klionsky, D. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8,** 445–544 (2016).

17. Mizushima, N. & Yoshimori, T. How to Interpret LC3 Immunoblotting. *Autophagy* 4–7 (2007). doi:10.4161/auto.4600

4.7. Acknowledgements

Dr Aurelio Teleman (German Cancer Research Center (DKFZ)) for generously sharing the anti-total S6K antibody.

Dr Thomas Neufeld (Department of Genetics, Cell Biology and Development, University of Minnesota) for sharing the GFP::Atg8a fly stock.

Dr Loren Looger and Jonathan Marvin (Howard Hughes Medical Institute (HHMI), Janelia Farm Campus) for sharing the arginine sensor, as well as unpublished data concerning its binding affinities and specificity.

Dr Ana Paula Elias for cloning of SLC7/CAT-cyto and SLC7/CAT-memb.

Bestgene for *Drosophila* embryo injection.

Vienna Drosophila Resource Center (VDRC) for RNAi stocks.

Chapter 5. Testing the involvement of autophagy related proteins in homeostatic feeding behaviour

5.1. Summary

Uncovering the genetics and cellular pathways underlying the regulation of behaviour is a fundamental step in understanding the nature of behaviour itself. Autophagy is an important degradation pathway in the cell, mediating both the bulk degradation of cytoplasmic components, as well as the selective degradation of proteins and organelles. Autophagy is negatively regulated by amino acids and mTOR activity. Moreover, in the previous chapter I showed that neuronal autophagic activity is correlated with an animals' accessibility to dietary protein and amino acids. In this chapter I describe experiments I designed to ask if this cellular degradation pathway plays an additional role in determining homeostatic feeding behaviour. I found no conclusive evidence that autophagy related genes play a role in the nervous system in mediating food choice behaviour. These results suggest that changes in neuronal autophagy activity alone may not be sufficient to drive behavioural changes associated with diets lacking protein.

5.2. Introduction

(Macro)autophagy was originally described as a bulk mechanism for gathering up and degrading proteins, organelles and other

cellular materials [1]. As such, it is difficult to imagine how this mechanism could be used to modify an animal's behaviour in a specific manner. However, since the initial discovery of autophagy several lines of evidence suggest the existence of different types of selective autophagic degradation pathways. Single proteins and various cellular structures such as protein aggregates, perioxisomes, ribosomes and mitochondria can be specifically engulfed by the growing autophagic vesicles [2]. This selective degradation has been shown to have several physiological consequences for the cell. For example, it has been shown that the selective degradation of the protein *highwire* at *Drosophila* larval neuromuscular junctions can regulate synaptic plasticity [3]. Also, in *C.elegans,* autophagy selectively degrades GABA receptors in the postsynaptic compartment to modify synapse strength [4].

Neuronal autophagic activity has previously been linked with behavioural modification. For example, in aging flies, autophagic activity decreases, and this is correlated with an increase in age-dependent memory impairment. Stimulation of autophagy can rescue this memory impairment [5]. It has also been shown that hypothalamic autophagy is important for regulating feeding behaviour, as mice mutant for one of the autophagy related proteins in agouti-related peptide (AgRP) neurons fail to show compensatory feeding after a period of starvation [6].

I hypothesised that animals may have developed a specific use of autophagy in neurons, perhaps as a 'nutrient sensing mechansim', and that this nutrient sensing ability could in turn lead to modifications within the neurons, perhaps through

selective degradation of proteins, which would in turn modify food choice and homeostatic feeding behaviour.

5.3. Material and Methods

Fly rearing, media and dietary treatments: Flies were reared in yeast-based food containing (per litre of water: 80g sugar cane molasses, 22g sugarbeet syrup, 8g agar, 80g corn flour, 10g soya flour, 18g yeast extract, 8ml propionic acid, 12 ml nipagin (15% in EtOH)). Upon emerging as adults, groups of 3-6 days old flies (15 females and 5 males) were transferred to fresh yeast based food for 3 days and then to 100mM sucrose for a further 3 days.

Fly stocks: *Atg1[GD16133], Atg13[CG27955, GC27956], Atg101[GD27815, GD27816], Atg6[GD22122, GD22123], CG11877[GD49372], Atg3[GD22455], Atg4[GD34843], Atg7[GD27432], Atg8a[GD43096, GD43097], Atg16[GD25651, GD25652].* All RNAi lines were from the Vienna Drosophila Resource Center GD library. *PblGal4* was a gift from M. Alenius. *w^{1118}; nSybGal42.1* was from the Vienna Drosophila Resource Center.

Sugar/yeast choice assay: Upon emerging as adults, groups of 3-6 days old flies (15 females and 5 males) were transferred to fresh yeast based food. After 72h, the flies were tested for nutrient choice. Two-choice color feeding preference assays were performed as described in Ribeiro and Dickson, 2010 [7]. Flies were given the choice between sucrose mixed with red colourant (20mM sucrose; 7.5mg/ml agarose; 5mg/ml Erytrosin B

(Sigma-Aldrich 198269); 10% PBS) or yeast mixed with blue colourant (10% yeast (SAF instant yeast); 7.5mg/ml agarose; 0.25mg/ml Indigo carmine (Sigma-Aldrich 131164); 10% PBS) medium. After visual inspection of the abdomen, each female fly was scored as having eaten sucrose (red abdomen), yeast (blue abdomen), or both (red and blue or purple abdomen) media. The yeast preference index (YPI) for the whole female population in the assay was calculated as follows: (nblue yeast – nred sucrose)/(nred sucrose + nblue yeast + nboth).

5.4. Results

5.4.1. Identification of autophagy related proteins as regulators of feeding behaviour

From a genome-wide RNA-mediated gene interference (RNAi) screen conducted by Carlos Ribeiro (data unpublished), three autophagy related genes (Atgs), *Atg1* (CG10967), *Atg7* (CG5489) and *Atg8a* (CG32672), were identified as candidate genes in the nervous system for regulating the yeast preference of flies. The screen was based on the assumption that flies have a neuronal mechanism to sense internal amino acid levels, and this mechanism drives compensatory changes in food choice behaviour in response to changing levels of internal amino acids. If a gene is required in the nervous system as part of this mechanism, then flies in which the gene is knocked down will fail to exhibit adaptive changes in feeding behaviour appropriate to nutrition levels. This initial observation, that expression of RNAi transgenes in the nervous system targeting three autophagy

related genes seemed to reduce the yeast preference index of flies was evidence for the role of this pathway in homeostatic feeding. This, combined with my findings presented in the previous chapter, correlating changes in autophagic activity with protein status of the animal, gives strong support to further investigate the role of the autophagic pathway in homeostatic feeding.

In order to confirm and expand this original screen data I tested whether the pan-neuronal expression of RNAi transgenes targeting multiple autophagy related genes would reveal behavioural phenotypes in food choice. Autophagy is a highly conserved process, and Zinn and Perrimon list 20 Atg's found in *Drosophila* with homologous mammalian counterparts [8] **(Figure 5.1A)**. Using the Vienna *Drosophila* Resource Center (VDRC) GD library [9], I selected eleven of these genes as targets for RNAi testing.

I tested adult females of the genotype *elav>'X' IR* (inverted repeat), aged between three and six days. In order to enhance the RNAi pathway, I simultaneously induced the over-expression of Dicer2 protein [9]. Flies were fed for three days on yeast-based food (YBF), followed by three days of 100mM sucrose, so to deprive the flies of protein. For behavioural testing flies were subjected to a 2-choice feeding assay. In this assay, flies are given the option of feeding from a 10% yeast solution with blue food colouring or a 20mM sucrose solution with red food colouring **(Figure 5.1B)**. The yeast preference index of these flies can then be calculated by scoring the colour of the fly's abdomen. Flies that have been kept on a rich diet containing

5.1

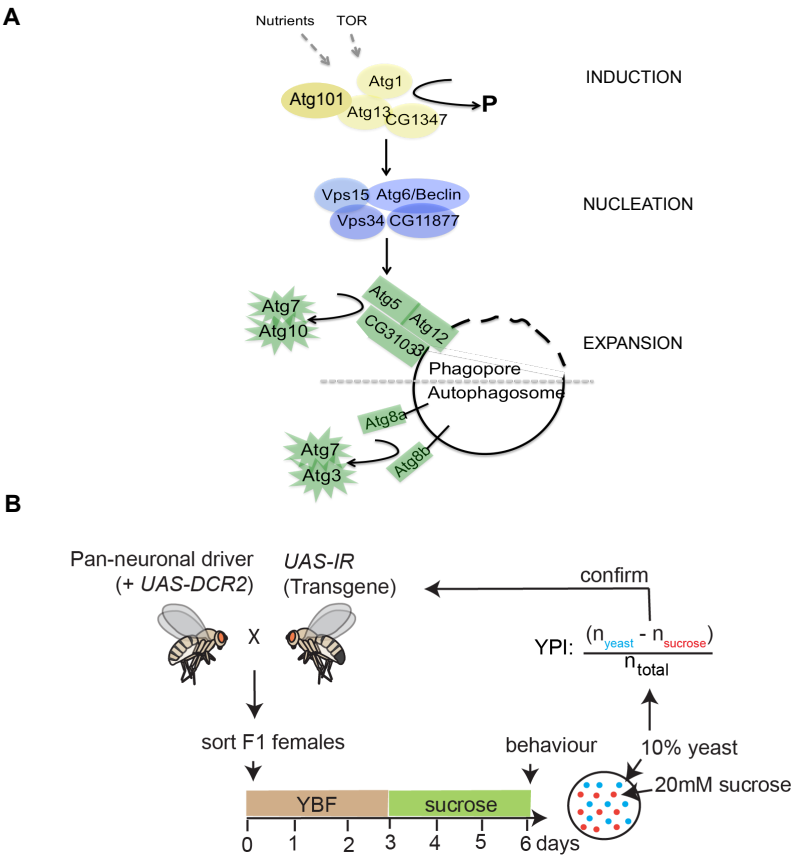


Figure 5.1. Testing the involvement of autophagy related proteins in feeding behaviour. **(A)** A diagram of the Atgs conserved in *Drosophila* and colour coded with the 3 steps of autophagy, initiation of autophagy (yellow), nucleation of the phagophore (purple) and expansion of the autophagosome membrane (green). **(B)** Schematic representation of the experimental setup designed to test the requirement for the Atgs in regulating food choice.

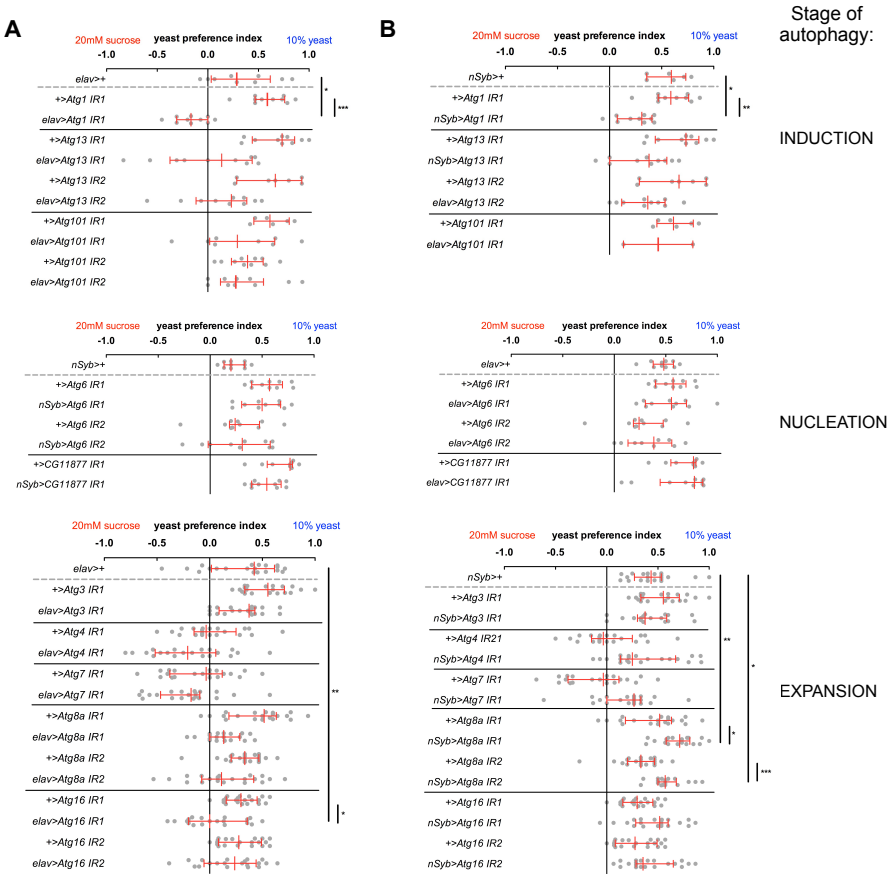


Figure 5.2. Results from the screen revealing Atg1 as a possible candidate for the regulation of homeostatic feeding. (A-B) Yeast preference index of flies kept on 100mM sucrose for 3 days prior to the behavioural assay**. (A)** Pan-neuronal expression (*elav-Gal4*) of hairpins (IRs) against Atgs. **(B)** Pan-neuronal expression (*nSyb-Gal4*) of hairpins (IRs) against Atgs. n=6-20 for all conditions (one condition had n=2); significance tested using Kruskal-Wallis test followed by Dunns multiple comparisons; error bars show median and IQR; significance only indicated in the instances where both controls differ from the knockdown; p<0.05*, p<0.01**, p<0.001***.

protein exhibit a low yeast preference. The yeast preference increases after flies are fed a food lacking protein [7]. Using this 2-choice colour assay I identified two RNAi lines that when expressed pan-neuronally reduced the yeast preference of flies **(Figure 5.2A)**, *Atg1*, identified in the original genome-wide screen, and *Atg16*. *Atg7* and *Atg8a* were also identified in the original screen. However, I found that the pan-neuronal expression of the transgene targeting *Atg7* gave a phenotype that matched that of the UAS line alone i.e. the phenotype was not specific to the pan-neuronal expression of the RNAi transgene. I also found no evidence to support the observation that knockdown of Atg8a reduced yeast preference in flies, flies in which *Atg8a* expression was reduced in the nervous system showed no reduction in yeast preference.

In an attempt to improve the likelihood of finding candidate genes, I also tested adult females of the genotype *nSyb>'X' IR.* This pan-neuronal driver is much stronger than *elav-Gal4.* From this round of screening the expression of the RNAi transgene against *Atg1* was again found to decrease yeast preference. The expression of the RNAi transgenes against *Atg8a* were found to increase the yeast preference of the flies **(Figure 5.2B)**. This is in contrast to the observations from the original screen.

Given the above results, *Atg1* was the top candidate for a gene that may be necessary in the nervous system to drive feeding behaviour modifications. *Atg1* plays a pivotal role in autophagy, acting as a kinase at the top of the autophagy pathway, phosphorylating downstream targets to activate autophagy in the cell.

As a follow up to the screen, I asked whether I could find a smaller set of neurons in which *Atg1* might be both necessary and sufficient to drive changes in feeding behaviour. The expression of the RNAi transgene targeting *Atg1* by the pan-sensory driver *pebbled-Gal4 (Pbl-Gal4)* [10] resulted in a decreased preference for yeast in the 2-choice colour assay **(Figure 5.3A)**. Suggesting that changes in autophagy in sensory neurons maybe be sufficient to drive behavioural changes in the fly upon protein deprivation. I also tested Gal4 lines that had been previously identified in a screen for neurons that when their activity was silenced would lead to a decrease in yeast preference (data unpublished). However, the expression of the RNAi transgene targeting *Atg1* by these Gal4 lines did not result in any changes in yeast preference **(Figure 5.3B)**.

5.5. Discussion

The observation that the pan-neuronal expression of an RNAi transgene targeting *Atg1* for RNAi mediated knockdown, leads to a reduction in yeast preference in protein deprived flies suggests this gene is necessary for flies to recognise their internal state and respond to it. Moreover, given that the knockdown of *Atg1* only in sensory neurons is sufficient to repress yeast preference in protein deprived flies, it is possible that autophagy in sensory neurons can drive homeostatic feeding behaviour. Taking into account results from the previous chapter where it was shown that autophagy activity increases with protein deprivation, these last results suggest that if autophagy cannot be increased in

5.3

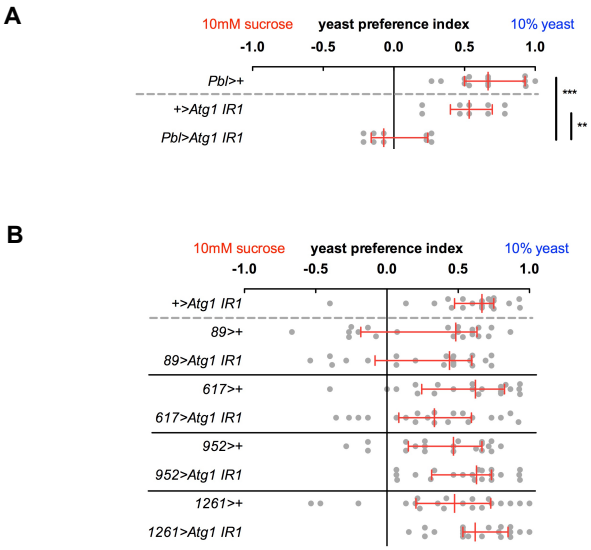


Figure 5.3. Atg1 may play a role in sensory neurons in regulating homeostatic feeding. (A-B) Flies were kept on 100mM sucrose for 3 days prior to the behavioural assay**. (A)** Pan-sensory (*Pbl-Gal4*) expression of *Atg1* IR1. **(B)** Expression of *Atg1* IR with Gal4 lines identified in a previous screen. n=9-20; significance tested using Kruskal-Wallis test followed by Dunns multiple comparisons; error bars show median and IQR; significance only indicated in the instances where both controls differ from the knockdown; p<0.01**, p<0.001***.

sensory neurons, then deprivation-induced changes in behaviour are suppressed.

However, after expressing RNAi transgenes against nearly all of the autophagy related proteins only one IR against one gene, *Atg1*, was found to consistently modulate feeding behaviour. Taking this into account, it is not possible to rule out off-target effects of the IR targeting *Atg1*. Further IRs against *Atg1* would need to be tested, preferably ones that do not overlap in target sequence with the original line identified, to verify the observations of this chapter. Alternatively, I would need to show that RNAi mediated knockdown of other autophagy related genes gave behavioural phenotypes in food choice. Perhaps I did not identify more autophagy related genes because of the lack of temporal specificity in the experimental design. I expressed the IRs for the entire development of the animal (*elav-Gal4* is known to be expressed in the adult fly, but also during development), and autophagy is known to play numerous roles through-out the developmental period, including during metamorphosis. Introducing temporal specificity maybe would alleviate complications caused by disruption of autophagy at early time points, and perhaps uncover behavioural phenotypes specific to adults. Furthermore, the reduction in yeast preference in Atg1 knockdown flies may not be specific to feeding behaviour – it may be a result of loss of motor or coordination abilities in these flies. Including other behavioural assays would be needed to control for the specificity of this phenotype.

Finally, it is clear *Atg1* plays a key role in autophagy. It has been characterised as the only serine/threonine kinase in the

autophagy signalling pathway. Furthermore, the Atg1-Atg13 complex formation is directly regulated by the kinase activity of mTOR in amino acid replete conditions [8]. However, *Atg1* is also known to play a key role outside of autophagy in axonal transport [11]. The dual function of this protein means it is difficult from the results presented in this chapter to convincingly state a role for autophagy in regulating feeding behaviour. Instead, perhaps changes in neuronal autophagy in response to dietary manipulations is fulfilling the more traditional role of 'recycling and reusing', buffering nutrients in neurons in times of need, and is not directly linked to changes in behaviour.

5.6. References

1. Schworer, C. & Mortimore, G. Glucagon-induced autophagy and proteolysis in rat liver: mediation by selective deprivation of intracellular amino acids. *Proc. Natl. Acad. Sci. USA* **76,** 3169–3173 (1979).

2. Kraft, C., Peter, M. & Hofmann, K. Selective autophagy: ubiquitin-mediated recognition and beyond. *Nat. Cell Biol.* **12,** 836–841 (2010).

3. Shen, W. & Ganetzky, B. Autophagy promotes synapse development in Drosophila. *J. Cell Biol.* **187,** 71–79 (2009).

4. Rowland, A. Presynaptic Terminals Independently Regulate Synaptic Clustering and Autophagy of GABAA Receptors in Caenorhabditis elegans. *J. Neurosci.* **26,** 1711–1720 (2006).

5. Gupta, V. K. *et al.* Restoring polyamines protects from age-induced memory impairment in an autophagy-dependent manner. *Nat. Neurosci.* (2013). doi:10.1038/nn.3512

6. Kaushik, S. *et al.* Autophagy in Hypothalamic AgRP Neurons Regulates Food Intake and Energy Balance. *Cell*

Metab. **14,** 173–183 (2011).

7. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

8. Zirin, J. & Perrimon, N. Drosophila as a model system to study autophagy. *Semin. Immunopathol.* 1–10 (2010).

9. Dietzl, G. *et al.* A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448,** 151–156 (2007).

10. Sweeney, L. B. *et al.* Temporal Target Restriction of Olfactory Receptor Neurons by Semaphorin-1a/PlexinA-Mediated Axon-Axon Interactions. *Neuron* **53,** 185–200 (2007).

11. Toda, H. *et al.* UNC-51/ATG1 kinase regulates axonal transport by mediating motor-cargo assembly. *Genes Dev.* **22,** 3292–3307 (2008).

5.7. Acknowledgements

Vienna Drosophila Resource Center (VDRC) for RNAi stocks.

Chapter 6. Identification of *beefeater* as a novel regulator of homeostatic feeding behaviour

6.1. Summary

The question of how the nervous system senses changing internal levels of amino acids and, in turn, how the animal responds to these amino acid changes is highly relevant in the field of feeding behaviour. Detection of amino acids and control of intake seems to take place both peripherally, along the gastrointestinal tract, as well as in the central nervous system of the animal. Since the central nervous system controls behaviour, the determination of nutrient sensing mechanisms in the nervous system is of specific importance in this context. In this chapter, I sought to determine whether amino acids could be driving the behavioural changes observed in food choice behaviour by directly acting in the nervous system. If the nervous system requires access to amino acids in order to modulate food choice behaviour, then manipulation of this access should result in behavioural phenotypes. To do this, I induced the expression in neurons of RNAi transgenes that target putative amino acid transporters, as annotated in the fly genome. I found one locus, previously annotated as *CG12531*, and which I renamed *beefeater*. The protein, beefeater, is localised to the lysosome and is both necessary and sufficient for the regulation of food choice. These results suggest lysosomal transportation of amino acids plays a key role in neuronal nutrient sensing, and is an essential component of the signaling pathway used by neurons

to regulate food choice, and as such, homeostatic feeding behaviour.

6.2. Introduction

An animal's control of food intake is regulated at multiple levels, including food selection, meal size, and number of meals. Food selection is regulated by the senses, olfaction and gustation [1 2]. The meal size is usually described as the parameter for post-ingestive regulation. Thus, during a meal the animal has direct feedback determining when to stop eating, and this could be because the animal has met its requirements or because the food source is not good [3]. Finally, animals not only regulate food intake during a meal, but over much longer periods of time, depending on their internal state. It has been shown that animals increase their food intake following a period of starvation or macronutrient deprivation [4]. A variety of satiety signals are produced by the animal in response to these behaviours. These are produced both peripherally, by the gastrointestinal tract and adipose tissue and centrally, in the nervous system. These signals emanate from the taste and smell of the food, food-induced stretching of the stomach, or the nutritional value of the food [5, 6]. With respect to the assessment of nutritional value, the concept of a 'nutrient sensor' is central. The nutrients that enter the body of the animal during feeding need to be detected, and in turn this information needs to be available to the nervous system in order for behavioural responses to be updated, for example termination of a meal. To explain this interaction between the

nervous system and the other tissues of the body, two hypotheses have been put forward. The brain could detect these nutrient changes indirectly: as recently described, in response to protein intake the fat body peptide female-specific independent of transformer (FIT) is release into the haemolymph, and acts on the brain to promote the release of *Drosophila* insulin-like peptide 2, which suppresses protein intake [41]. Alternatively, nutrients could act directly on the nervous system, and this could directly modulate feeding behaviour. In support of this, it has been shown that neurons of *Drosophila* larvae can respond directly to imbalanced mixes of amino acids [3 7]. Furthermore, studies in rodents showed that orexin/hypocretin neurons can be stimulated by nutritionally relevant mixtures of amino acids [8].

In the case where the brain responds directly to nutrients, the candidate nutrient sensing pathways are the highly conserved mechanistic Target Of Rapamycin (mTOR) pathway and the General Control Nonderepressing 2 (GCN2) pathway. Both pathways are known to respond to nutrient signals, and most cell types use this nutrient information to control metabolic processes such as growth. Moreover, there is growing evidence for the involvement of these pathways in the nervous system in the regulation of feeding behaviour. Modulation of neuronal mTOR signalling affects the yeast preference of flies in 2-choice colour assays in adult *Drosophila* [4], whilst modulation of neuronal GCN2 signalling blocks the ability of *Drosophila* larvae to reject amino acid imbalanced diets [3].

In chapter 4 I described my findings that the activity of the nutrient sensitive mTOR pathway and autophagy is modulated in

response to dietary manipulations of protein and amino acids in the heads of flies. Furthermore, I showed that the essential amino acid arginine also seems to fluctuate in the nervous system following manipulation of dietary protein and amino acids. These findings are supported by recently published work showing that removal of a single dietary amino acid reduces the levels of that amino acid in head preparations in flies [9]. Altogether, these results suggest that neuronal amino acid levels could be affected by changes in amino acids levels in the flies' diet. In order to ask whether changes in neuronal amino acid levels are capable of driving food choice behaviour, I performed a pan-neuronal RNAi screen of predicted amino acid transporters within the Solute Carrier (SLC) families. These transporter proteins could be described as the 'gate-keepers' to the cell, regulating the flux of amino acids across the cells membranes. Whilst some of these proteins have previously been described as playing important roles in transport and nutrient sensing and signalling [10] [11] [12] [13], many of the genes in the SLC families are uncharacterised. Working with one candidate gene identified in this screen, *CG12531*/*beefeater*, I went on to ask what the underlying mechanism is of this gene in the nervous system, and how it exerts its effects on feeding behaviour.

6.3. Materials and Methods

Fly stocks: All RNAi lines were from the Vienna Drosophila Resource Center (VDRC) KK library, GD library, National Institute of Genetics (NIG). *CG9073[KK103240]* is the non neuronal control used for the KK library. *slifanti* was a gift from

P. Leopold. *Df(3L)773, P{GSV6}path*^{GS11111}*, Df(1)Exel6253, beefeater*^{MI13892}*,* intergenic^{MI00606} from Bloomington Drosophila Stock Center (BDSC). *GFP::LAMP1* was a gift from H.Kramer.

Holidic media: Holidic media were made according to Piper et al., 2014 and Piper et al 2017 [14].

Sugar/yeast choice assay: Two-choice color feeding preference assays were performed as described in Ribeiro and Dickson, 2010 [4], and as outlined in previous chapters.

FlyPAD Monitoring of Feeding Behavior: As described in previous chapters and in Itskov et al., 2014 [15].

RNA Extraction and Quantitative Real-Time PCR: Done as outlined in Chapter 4. DNA sequence of extra primers listed below:

CG3424_fwd – 5' CGCATCCAACAACCGATAAT 3'
CG3424_rvs – 5' GGTGGAGAAGATTCCCATGA 3'
CG3424_fwd2 – 5' GGGATTGATCTTCCCGGTGGT 3'
CG3424_rvs2 – 5' CCTTGATGGCTGCCTGGGT 3'
CG12531_fwd – 5' TGAGGGCCGAGATACAAAAG 3'
CG12531_rvs – 5' GCACCCTGAACTTGACCTTC 3'
CG12531_fwd2 – 5' GTATGGATGTCGCTGGGATT 3'
CG12531_rvs2 – 5' CGACGTTTTTGCTGTAGTCG 3'
Dilp2_fwd – 5' GTATGGTGTGCGAGGAGTAT 3'
Dilp2_rvs – 5' TGAGTACACCCCCAAGTAAG 3'
Dilp5_fwd – 5' TCAATTCAATGTTCGCCAAA 3'
Dilp5_rvs – 5' TGTGGTGAGATTCGGAGCTAT 3'
Sarah_fwd – 5' AAAAACGCCAGCCATGTCCGA 3'
Sarah_rvs – 5' TGCATTGGCCGCGTCAGCTT 3'

aTub84B_fwd – 5’ GCCGGCAGTTCGAACGTAT 3’
aTub84B_rvs – 5’ ACCAGCCTGACCAACATGGA 3’

Generation of transgenic lines:

shmiRs: 21 nucleotide sequences were designed using www.dkfz.de/signalling/e-rnai3/ targeting *pathetic* and *beefeater*. These same 21nt sequences were scrambled individually to use as control sequences. Cloning was performed as previously described by Jian-Quan Ni and Norbert Perrimon for the Transgenic RNAi project (Harvard Medical school). Oligonucleotides (Sigma) were annealed at a final concentration of 1μM in 80μl of annealing buffer (10mM Tris-HCl, pH7.5, 0.1M NaCl, 1mM EDTA). The mix was incubated at 95°C for 5 minutes, then allowed to cool for 1hr at room temperature. This DNA fragment with NheI and EcoRI overhangs was directly cloned into VALLIUM20, linearized by NheI and EcoRI. Ligation was done with 40ng of backbone and 6μl of annealing product, at 16°C for 1hr. Following transformation, colony PCR was performed, and the final colonies were sent for sequencing. DNA sequences of the oligonucleotides are listed below:

Path_top:
5’CTAGCAGTGCGTGACCCTTATTTATATGCTAGTTATATTCA
AGCATAATATAAATAAGGGTCACGCCCGCG3’
Path_bottom:
5’AATTCGCGGGCGTGACCCTTATTTATATTATGCTTGAATAT
AACTAGCATATAAATAAGGGTCACGCACTG3’
CG12531_top:

5’CTAGCAGTCCCAGGACGGCTTGATATTCATAGTTATATTCA
AGCATAAATATCAAGCCGTCCTGGGCCGCG3’
CG12531_bottom:
5’AATTCGCGGCCCAGGACGGCTTGATATTTATGCTTGAATA
TAACTATGAATATCAAGCCGTCCTGGGACTG3’

UAS-CG12531/beefeater: Full length *CG12531/beefeater* was generated synthetically from CG12531-RA coding sequence plus some extra sequence before the start codon: TCGTAACTGTTGAGGGCCGAGATACAAAAGCGCA
And after the stop codon:
GCACAGAGACCCAGTTGCAGTCCCAGTCCCTGTCCCTGTCC
CAGTTACCATTCCAA
Gateway sequences were added for cloning purposes and ECORV restriction sites. This construct was originally designed to be RNAi insensitive and in the target regions of IRKK and IR2 the triplet code has been silently mutated taking into account the codon bias of *Drosophila.* This oligonucleotide was cloned by gateway cloning into pUASgattB (gift from Basler lab, Zurich).
To add the mCherry tag a PCR was done using the initial oligonucleotides from the N terminus gateway sequence to the C terminal of CG12531 with an extra sequence containing the linker region for the mCherry:
GGAGGTTCCGGTGGAAGCGGAGGTAGCGGCGGATCC
And a second PCR from the linker region, including the mCherry sequence and gateway sequence. These two products were purified and a second PCR performed to generate the final product, which was cloned into pUASgattB (gift from Basler lab, Zurich).

Immunostaining and imaging: Female flies were dissected in PBS and fixed in PBS containing 4% PFA for a further 20-30 minutes. After fixation, the brains were incubated in blocking solution (PBS containing 10% Normal goat serum and 0.3% Triton-X 100) for 4 hours. The brains were then incubated for 72 hours at 4°C in primary antibody solution (PBS containing 5% normal goat serum and 0.3% Triton-X 100). The primary antibodies used in the present study were the following: rabbit anti-GFP (1:6000, torrie Pines), mouse anti-brp/NC82 (1:20, DSHB). After incubation with the respective primary antibodies, brains were washed 3 times 15 minutes in PBST, and then over-night in PBST. The brains were then incubated for another 72 hours in secondary antibody solution (PBS containing 5% normal goat serum and 0.3% Triton-X 100). The secondary antibodies, conjugated with either Alexa-488 or Alexa-647 (1:500, Invitrogen). Finally, the brains were washed 2 times in PBST, and a final time in PBS before mounting in Vectashield (VectorLaboratories) and observed under a confocal laser-scanning microscope LSM 710 (Zeiss). Image processing and analysis were done with Fiji (National Institutes of Health) and Photoshop CS5 (Adobe).

For cuticle preparations, adult female flies were washed in 96% ethanol. Legs, wings, and heads of adult females were removed and mounted in 70% glycerol for immediate imaging under a confocal laser-scanning microscope LSM 710 (Zeiss). Image processing and analysis were done with Fiji (National Institutes of Health) and Photoshop CS5 (Adobe).

Protein extraction, SDS-PAGE and Western analysis: P-Thr398 dS6K blots and Atg8a blots done as outlined in Ch 4**.**

6.4. Results

6.4.1. A targeted pan-neuronal screen to identify new regulators of food choice

To determine if neurons require access to amino acids in order to effectively drive changes in food choice I screened members of the Solute Carrier Transporters (SLCs) with sequence similarity to annotated amino acid transporters, for changes in food choice behaviour. A previous report describes 12 putative amino acid transporter families in mammals, 9 of which are present in insects [16]. I compiled a list of these transporters using the data from several references [16,17,18] **(Table 6.1)**, and cross-referenced it against Flybase data to produce a final list comprising 53 putative amino acid transporters. More than half of the transporters on this list had RNAi transgenic lines available in the KK library of the Vienna *Drosophila* Resource Center (VDRC) (29 UAS-IR lines, IR, inverted repeat) **(Figure 6.1A)**. I first tested if flies expressing these RNAi transgenes would be healthy enough for behavioural experiments. I crossed the RNAi transgene lines to female flies containing the pan-neuronal driver *elav-Gal4* and *UAS-Dicer2* (for more efficient gene silencing). From these lines, 27 were deemed viable for behavioural studies (expression of the RNAi transgene targeting *CG2791* was embryonic lethal and flies expressing the RNAi transgene targeting *CG1732* were too sick to be used in behavioural experiments). Briefly, almost no line was healthy enough for behaviour when crossed to the pan-neuronal driver *nSyb-Gal4*: some were lethal but many exhibited

Table 6.1

SLC FAMILY	GENE	VDRC number	*elav>IR*	*nSyb>IR*
SLC1	CG3747/Eaat1	109401	viable	sick
	CG3159/Eaat2	104371	viable	viable
SLC3	CG2791	108365	lethal	lethal
SLC6	CG1732/GAT	106638	sick	viable
	CG8380/DAT			
	CG4545/dSerT			
	CG5549			
	CG7075	102776	viable	sick
	CG5226			
	CG10804			
	CG15444/ine			
	CG15279	108759	viable	sick
	CG1698	101947	viable	strong wing phenotype
	CG4476	109677	viable	strong wing phenotype
	CG8850	104098	viable	sick
	CG15088	109791	viable	weak wing phenotype
	CG3252	106027	viable	pupal lethal
	CG3897/blot			
	CG8291			
	CG13796			
	CG31904			
	CG13793			
	CG13795			
	CG13794			
SLC7	CG11128/slimfast	110425	viable	viable
	CG12317/Jhl-21	108509	viable	strong wing phenotype
	CG9413			
	CG6070/gb			
	CG1607	105677	viable	viable
	CG3297/mnd	110217	viable	viable
	CG7255	107802	viable	weak wing phenotype
	CG12531	105771	viable	strong wing phenotype
	CG13248	102635	viable	strong wing phenotype
	CG5535	107030	viable	weak wing phenotype
SLC15	CG44402/OPT1			
SLC17	CG9887	104324	viable	
SLC32	CG13646/mah			
	CG8394	103568	viable	pupal lethal
SLC36	CG3424/pathetic	100519	viable	strong wing phenotype
	CG1139	102363	viable	viable
	CG7888			
	CG13384	106698	viable	viable
	CG8785			
	CG16700			
	CG4991	108419	viable	weak wing phenotype
	CG32079	104454	viable	viable
	CG12943	107119	viable	sick
	CG6327			
	CG32081	107023	viable	sick
SLC38	CG30394			
	CG13743	110773	viable	sick
	CG5262			
	CG3039			

Table 6.1. List of putative amino acid transporters. (Columns left-right) The SLC family each predicted transporter belongs to. The gene name. The identification number of the RNAi transgenic line available in VDRC/KK library. The phenotype following the induction of expression of the RNAi transgene by the pan-neuronal driver *elav-Gal4.* The phenotype following the induction of expression of the RNAi transgene by the pan-neuronal driver *nSyb-Gal4.*

6.1

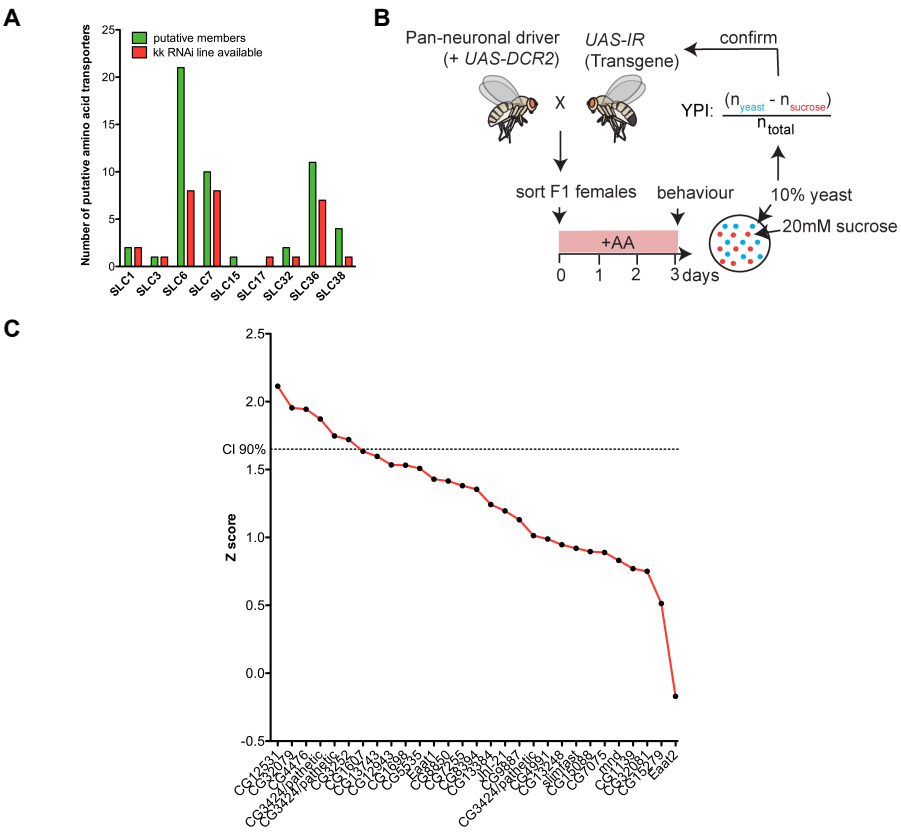


Figure 6.1. A pan-neuronal screen of putative amino acid (AA) transporters. **(A)** 9 of the 12 Solute Carrier (SLC) transporter families are conserved in *Drosophila*. For the screen the KK library from the Vienna Drosophila Resource center was used. RNAi lines (IRs) were available covering genes in all families. **(B)** Schematic of the screen. The pan-neuronal driver *elav-Gal4* was used to drive the expression of IRs against putative amino acid transporters. Adult female flies were prefed holidic media with amino acids, and after three days their yeast preference was tested in a 2-choice colourimetric feeding assay. The yeast preference index was scored, and candidate genes retested. **(C)** Results of the screen shown as a z-score of the yeast preference index. Cut-off was at a confidence interval (CI) of 90% for identification of candidate transporters playing a role in feeding behaviour.

a phenotype where the flies were unable to inflate their wings. This phenotype has been associated with the annotated insertion site in the KK library, and is thought to be the result of dominant-Gal4-dependent toxicity [19]. I therefore decided only to use the *elav-Gal4* driver for screening.

To perform the behavioural assay I used adult females. Female flies that have been kept on a rich diet containing protein or amino acids exhibit a low yeast preference that increases after a being fed a food lacking protein or amino acids [4, 9, 14, 20]. Females compared to males exhibit very different responses to dietary yeast deprivation in that their homeostatic response of increasing yeast preference is very fast, happening over a period of three days instead of the ten days defined for males [4]. Given this I hypothesised that any genetic manipulation of amino acid availability would have a greater effect in females than in males. For the screen, I hypothesised that if the nervous system required access to amino acids to drive changes in feeding behaviour I should see deviations in yeast preference in flies where the putative amino acid transporter is knocked down.

Age controlled, adult females of the genotype *elav>'X' IR* were collected and fed a holidic medium [20] containing amino acids for three days prior to behavioural testing **(Figure 6.1B)**. I decided to use synthetic food as the prefeeding treatment for this screen because of the stability of the food over successive batches prepared, and importantly because of the specificity it offers in later experiments when determining roles for macronutrients in behaviour, i.e., amino acids and sugars. For the behavioural testing I used a 2-choice colourimetric feeding assay. In this assay flies are given the option of 10% yeast with blue food

6.2

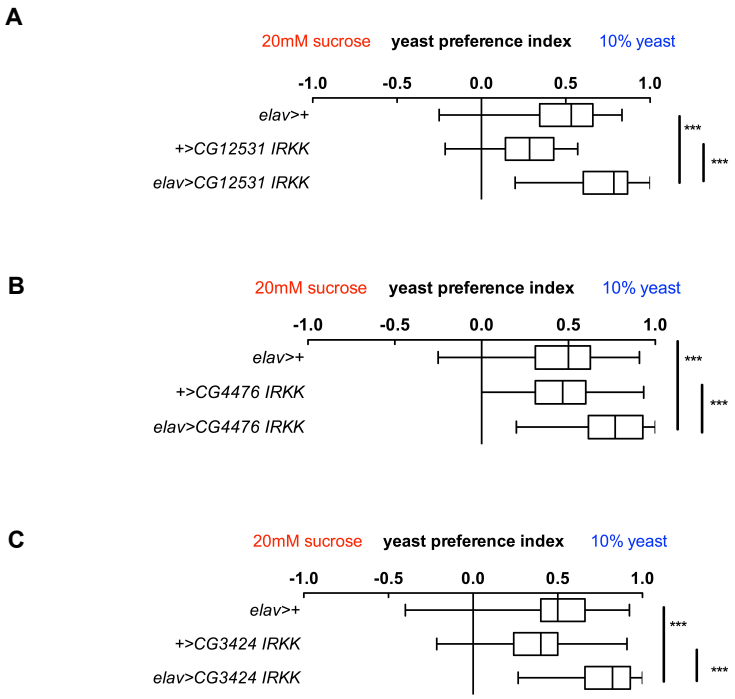


Figure 6.2. Three putative AA transporters act in the nervous system to regulate food choice. (A-C) The preference for yeast (higher yeast preference index, YPI) or sugar (lower YPI) of flies pan-neuronally expressing an IR targeting a putative AA transporter was scored following multiple retests after the screen. n=43-64 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; boxes show median and IQR, and whiskers show minimum/maximum values; $p<0.001$***.

6.3

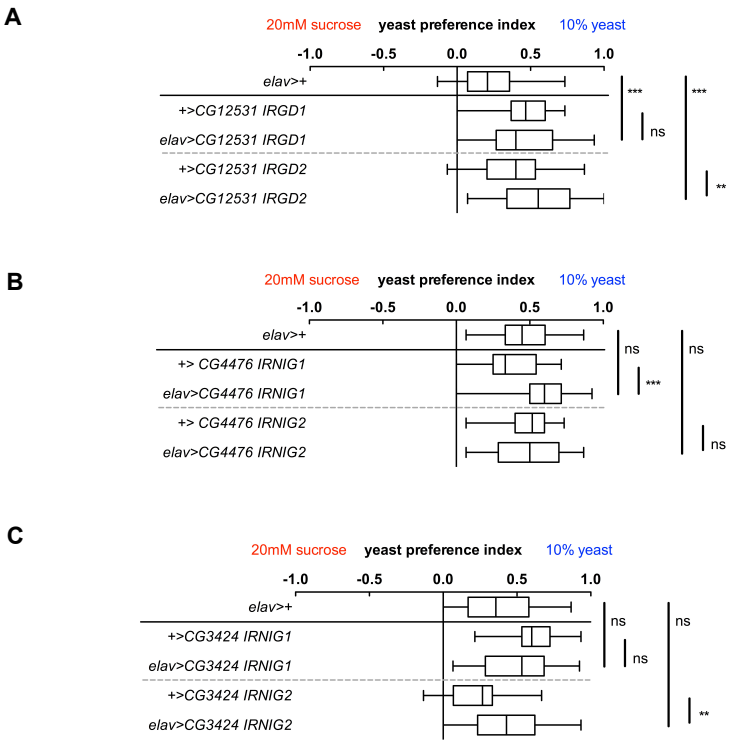


Figure 6.3. Testing additional IRs against the three AA transporter candidates. (A-C) The yeast preference index of flies pan-neuronally expressing additional IR's targeting the three candidate AA transporters identified in the screen. n=26-60 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; boxes show median and IQR, and whiskers show minimum/maximum values; p>0.05 ns, p<0.01**, p<0.001***.

colouring and 20mM sucrose with red food colouring. The yeast preference index of these flies can then be calculated by scoring the colour of the flies' abdomen.

Six RNAi lines produced flies with an increase in yeast preference above a 90% confidence interval (z-score of 1.65) **(Figure 6.1C).** Interestingly, no RNAi-induced LoF was able to cause a strong decrease in yeast preference in flies. The 6 RNAi lines increasing yeast preference were re-tested, and three of these loss-of-function (LoF) experiments resulted in flies with a robust and reproducible increase in yeast preference compared to controls, *CG12531, CG4476* and *CG3424* **(Figure 6.2A, B and C)**. Following the identification of these three candidate genes I tested for LoF phenotypes with all available RNAi transgenes for these genes. I found that the increased yeast preference caused by the loss-of-function of *CG12531* can also be induced by the expression of one independent RNAi transgene of the two that are available in the VDRC/GD library **(Figure 6.3A)**. No independent RNAi transgenes targeting the other two genes, CG4476 and CG3424, were found to give food choice phenotypes **(Figure 6.3B and C)**.

Amongst the list of initially tested putative amino acid transporters was the previously characterised gene, *CG11128/slimfast* [11]. This gene has been shown to play a nutrient sensor role in the fat body of *Drosophila* controlling the growth of the fly. Although it was not a strong candidate after the screen (z-score 0.98), I decided, given its importance in the literature, to retest it. Indeed, rigorous retesting of the line showed that RNAi transgene expression results in an increase in yeast preference **(Figure 6.4A)**. However, no other RNAi line

was found that could conclusively reproduce the phenotype expected for *slimfast* **(Figure 6.4B)**. Further work would need to be done to determine if this gene does indeed play a role in guiding food choice in the nervous system of the adult fly.

In conclusion, screening of predicted amino acid transporters using RNAi mediated knockdown, revealed four candidate genes that seem to be necessary for food choice in adult female flies, *CG12531, CG4476, CG3424 and CG11128.* For one of these genes, *CG12531*, I found two independent RNAi transgenes, strengthening the argument for a role of this gene in the nervous system in mediating food choice.

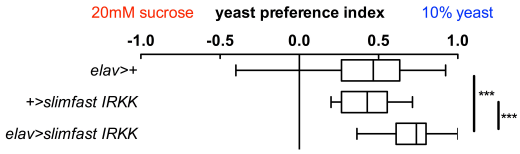
6.4.2. CG3424/pathetic plays a role in nervous system in the regulation of food choice

One of the candidate genes of the screen was *CG3424/pathetic.* This gene has been characterised as being required for growth in the fly, although not through bulk transport of amino acids, but instead by binding to amino acids. Furthermore, it appears to regulate the conserved nutrient sensing mTOR pathway [10, 21]. Regulation of feeding behaviour by the nervous system has also been implicated as relying on mTOR pathway activity [4, 22]; given this I decided to investigate the food choice phenotype of this gene further.

First, I addressed whether the increase in yeast preference was due to a modulation of sucrose or yeast feeding. Whilst the 2-

6.4

A



B

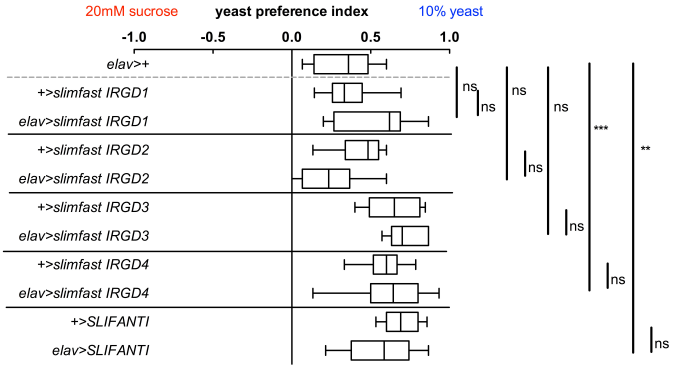


Figure 6.4. Neuronal *slimfast* may be also regulating feeding behaviour. (A) The YPI of flies pan-neuronally expressing an IR against *slimfast*. **(B)** The YPI of flies expressing additional IRs targeting *slimfast*. n=10-25 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; boxes show median and IQR, and whiskers show minimum/maximum values; p>0.05 ns, p<0.01**, p<0.001***.

choice colourimetric feeding assay used for screening is reasonably high throughput, and gives a good 'end-point' reading of preference change in flies between sucrose and yeast, it cannot tell us whether the fly is changing specifically sucrose or yeast feeding or a combination of both. To do this I employed a second behavioural assay, the Fly Proboscis and Activity Detector (FlyPAD) [15], with a 2-choice setup. This behavioural monitoring system uses capacitive-based measurements to detect the physical interaction of individual flies with a food source, and allows for the individual quantification of sucrose and yeast feeding behaviour in a time dependent fashion **(Figure 6.5A)**. These physical interactions of the fly with the food are highly correlated with actual food intake by the fly, herein termed 'sips'. In this assay, protein-fed and protein or amino acid-deprived flies feed on both yeast and sucrose. When subject to protein/amino acid deprivation, flies increase the total number of sips on both yeast and sucrose **(Figure 6.5B and C)**. In contrast, flies in which *CG3424*/*pathetic* has been knocked down pan-neuronally, using the RNAi line identified in the screen described previously, increase their number of sips specifically on yeast **(Figure 6.6A)**. Interestingly, sucrose feeding remains unchanged **(Figure 6.6B)** suggesting *pathetic* specifically mediates protein feeding. I used qRT-PCR to confirm the reduction in expression levels of *pathetic* transcript in the knockdown flies and found it to decrease approximately ~10% compared to wild-type transcript levels of the more conservative control flies **(Figure 6.6C)**. This reduction is extremely small and fails to achieve statistical significance against one of the genotype controls. However, this could be due *pathetic* being expressed outside of the nervous system in other head tissues

6.5

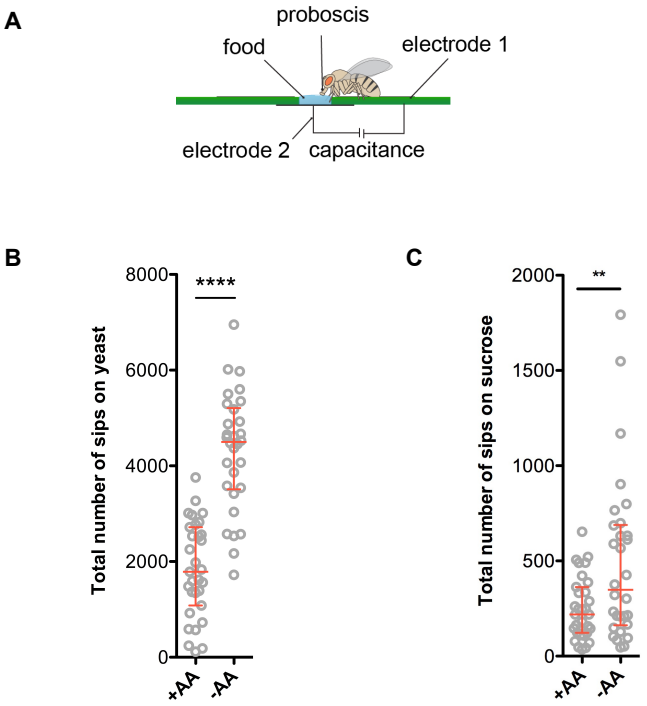


Figure 6.5. Amino acid deprivation increases feeding on yeast and sucrose. **(A)** Schematic of the flyPAD behavioural setup. Single female flies are loaded into arenas, each with two spots of food, 10% yeast and 20mM sucrose. Flies are allowed to feed for 1 hour. **(B)** Total number of sips on yeast of w^{1118} flies after prefeeding on the holidic diets indicated on the x-axis. **(C)** Total number of sips on sucrose of w^{1118} flies after prefeeding on the holidic diets indicated on the x-axis. n=30-31 for all conditions; Mann-Whitney test; data shown as median and IQR; p<0.01**, p<0.0001***.

6.6

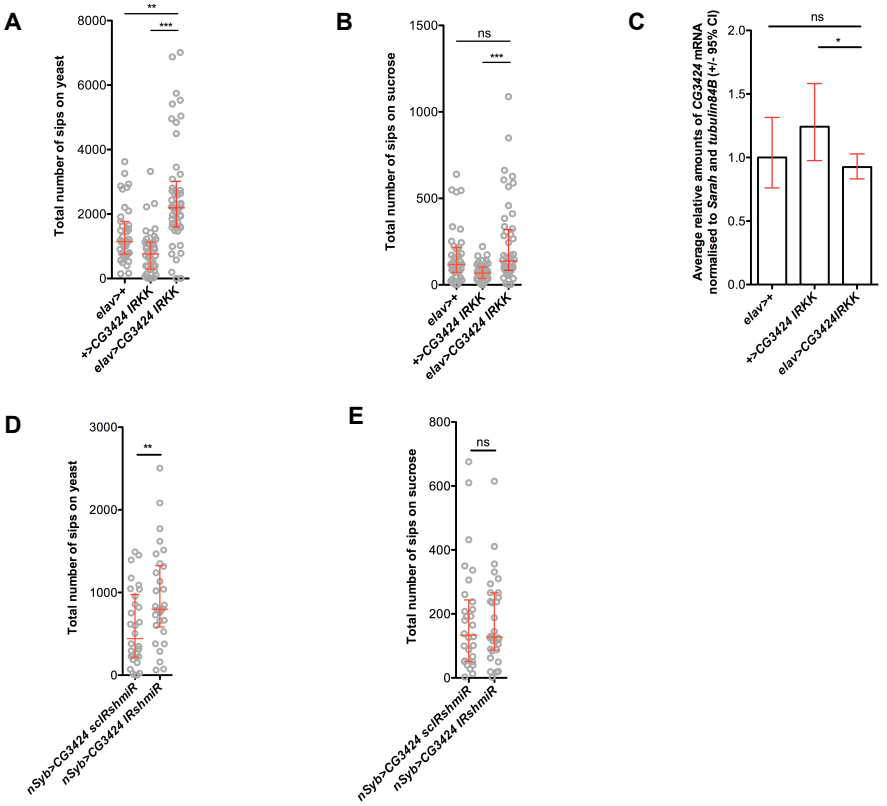


Figure 6.6. Pan-neuronal knockdown of *CG3424/pathetic* increases yeast feeding whilst leaving sucrose feeding unchanged. (A) Total number of sips on yeast of flies expressing an IR targeting *CG3424/pathetic* (IRKK). **(B)** Total number of sips on sucrose of flies expressing an IR targeting *CG3424/pathetic* flies (IRKK). **(C)** *CG3424/pathetic* transcript levels normalised to *Sarah* and *tubulin84B* as obtained by qRT-PCR. n=3, t-test; data shown as mean, error bars show 95% CI; p>0.05 ns, p<0.05*. **(D)** Total number of sips on yeast of flies expressing an additional hairpin (IRshmiR) targeting *CG3424/pathetic*. **(E)** Total number of sips on sucrose of flies expressing an additional hairpin (IRshmiR) targeting *CG3424/pathetic*. **(A, B, D and E)** n=30-31 for all conditions; Mann-Whitney test or Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; p>0.05 ns, p<0.01**, p<0.0001***.

and the non-neuronal signal masking the neuronal signal. In addition, I designed a second RNAi transgene according to the protocol outlined by Haley and colleagues [23] to confirm the LoF phenotype for *pathetic*. Haley and colleagues designed 21 nucleotide sequences to target genes of interest incorporated into a microRNA stemloop (shmiR), minimising the off-target effects associated with traditional long hairpin IRs. This also allowed me to design a control RNAi line, in which the 21nts targeting *pathetic* were scrambled to target no gene (scshmiR). The loss-of-function experiment in which these shmiRs were expressed in the nervous system resulted in a specific increase in the number of sips on yeast **(Figure 6.6D and E)**. Taken together, the data indicate first, that the increased yeast preference observed upon expression of the RNAi transgenes specifically results from the down-regulation of *CG3424/pathetic* and is not an artifact of RNAi off-targeting; second, that *CG3424/pathetic* appears to regulate the intake of protein alone, and has no role in sucrose feeding behaviour.

To strengthen the argument for the role of *pathetic* in food choice, I also asked if a deficiency, *Df(3L)BSC773*, covering the *pathetic* locus could enhance the RNAi phenotype. However, the combination of this deficiency with RNAi transgene expression did not increase the number of sips on yeast of the flies when compared to the expression of the RNAi alone **(Figure 6.7A, and B)**. Notably, flies heterozygous for the deficiency exhibit a decrease in number of sips, strongly suggesting that this deficiency extends over a gene in the chromosome which could have a dominant effect on the phenotype – not necessarily surprising given the pleitropic effects associated with deficiencies.

Finally, I tested whether *pathetic* is also sufficient to regulate yeast feeding. To do this, I induced the overexpression of *pathetic* pan-neuronally and asked if this reduced yeast feeding in flies. Using a transgenic line available from the GS library in combination with *nSyb-Gal4*, I tested the flies using the 2-choice colourimetric assay **(Figure 6.8A)**. Surprisingly, flies with a *pathetic* gain-of-function (GoF) did not decrease their preference for yeast and instead showed an increase in yeast preference **(Figure 6.8B)**. However, the apparent contradiction to our previous conclusions extensively supported by the results in LoF experiments is not uncommon in over-expression experiments, where any variation in terms of amount of a protein in the cell can be detrimental to the cellular process it controls. Nevertheless, I confirmed the over-expression of *pathetic* by qRT-PCR, and found it to be approximately doubled **(Figure 6.8C).**

Together these results indicate that *pathetic* plays a role in the nervous system in food choice of adult *Drosophila*. Interestingly, experimental evidence indicates *pathetic* acts as a transceptor, binding amino acids with high affinity, and genetically interacts with components of the mTOR pathway to regulate growth in *Drosophila* [10]. Furthermore, *pathetic* was shown to be required for dendrite growth in *Drosophila* larvae sensory neurons [24]. Given this data, it will be interesting in future work to unravel the molecular mechanisms underlying the neuronal *pathetic* function in food choice.

6.7

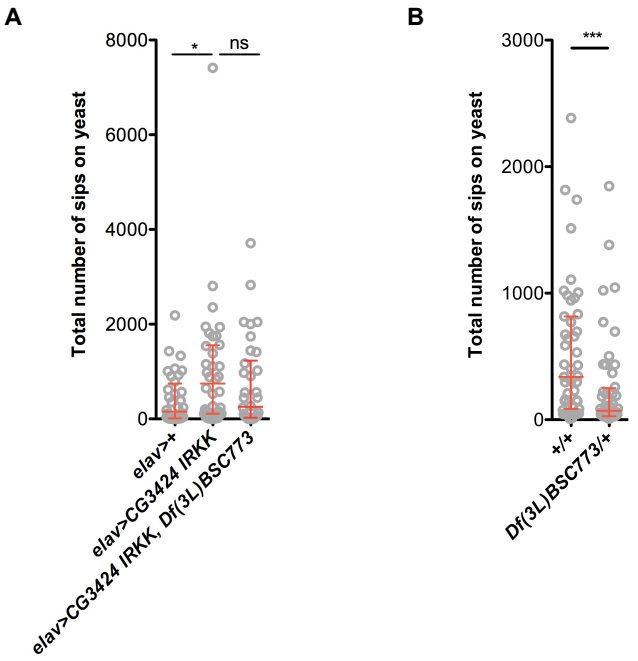


Figure 6.7. The loss of function phenotype of *CG3424/pathetic* is not enhanced by a deficiency spanning the *CG3424/pathetic* locus. **(A)** Total number of sips on yeast of flies pan-neuronally expressing an IR targeting *CG3424/pathetic* (IRKK) together with a deficiency spanning the *CG3424/pathetic* locus (*Df(3L)BSC773*). **(B)** Total number of sips on yeast of flies heterozygous for the deficiency. n=34-48 for all conditions; Mann-Whitney test or Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; $p>0.05$ ns, $p<0.05$*, $p<0.0001$***.

6.8

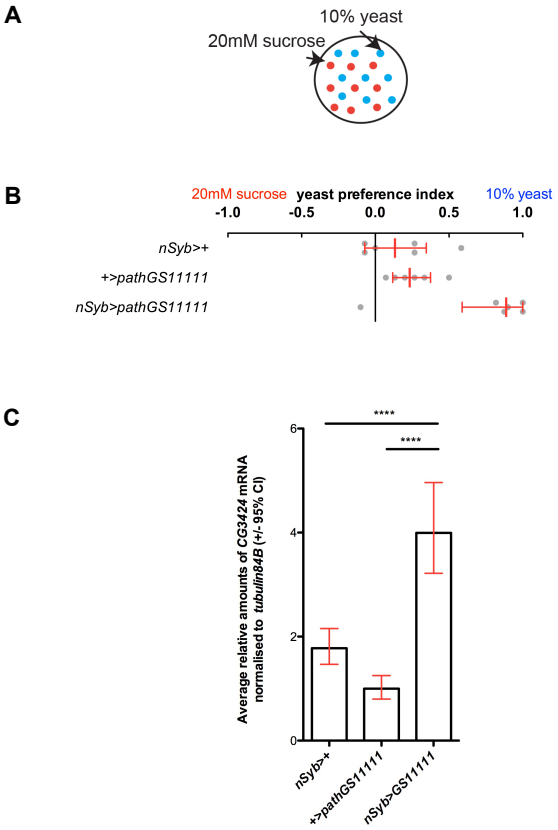


Figure 6.8. Effects of the over-expression of *CG3424/pathetic* on feeding behaviour. **(A)** The 2-choice colourimetric assay used for these experiments. 15 females and 5 males are loaded into each plate for 2 hours in the dark. The dietary choice is between 10% yeast with blue food colouring, and 20mM sucrose with red food colouring. **(B)** The YPI of flies pan-neuronally (*nSyb-Gal4*) over-expressing *CG3424/pathetic* (GS11111). Boxes show median and IQR, and whiskers show minimum/maximum values. **(C)** *CG3424/pathetic* transcript levels normalised to *tubulin84B* as obtained by qRT-PCR. n=3; significance tested using ANOVA; data shown as mean, error bars show 95% CI; p<0.0001****.

6.4.3. *CG12531/beefeater* is necessary and sufficient for the regulation of homeostatic feeding behaviour

The top candidate gene identified following the 2-choice screen looking for regulators of food choice was *CG12531*. This screen and following LoF experiments identified two independent RNAi transgenes targeting *CG12531*, that when expressed in the nervous system consistently increased yeast preference in female flies. Given the potential suggested by this data, I went on to ask whether changes in yeast or sucrose feeding were driving the increased yeast preference in these flies using the flyPAD behavioural setup. To do this I induced the pan-neuronal expression of the RNAi transgene found in the screen, IRKK **(Figure 6.9A)** to down-regulate the expression of *CG12531* in the fly. The resulting LoF gave a strong increase in the number of sips on yeast **(Figure 6.9B)**, whilst leaving sucrose feeding unchanged **(Figure 6.9C)**. The previously identified RNAi line from the VDRC/GD library **(Figure 6.3A)** is not controlled for in terms of the RNAi transgene insertion site in the genome. For the sake of precision, I therefore designed my own second RNAi line targeting *CG12531,* IRshmiR **(Figure 6.9A)**, along with an appropriate control, as an independent RNAi transgene to test in the flyPAD setup. When the expression of IRshmiR is driven by *elav-Gal4* there is no LoF phenotype (data not shown). However, when IRshmiR expression is driven by the stronger *nSyb-Gal4*, there is a significant increase in the number of sips on yeast **(Figure 6.9D)**. Furthermore, analysis of the sucrose feeding shows that the LoF has no effect on the respective number of sips **(Figure 6.9E)**. Quantification by qRT-PCR confirms that the

6.9

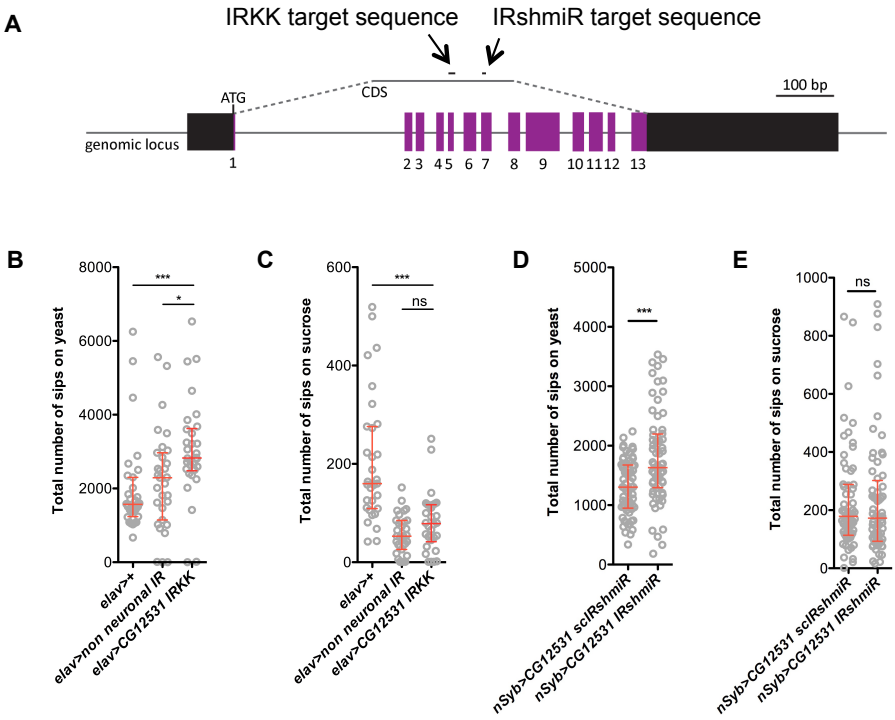


Figure 6.9. Pan-neuronal knockdown of *CG12531* increases yeast feeding whilst leaving sucrose feeding unaffected. **(A)** Gene diagram of *CG12531*. UTRs in black, exons are in purple and numbered 1-13. Exact positions of IRs targeting *CG12531* are marked in CDS. **(B-E)** Food intake was assessed using the flyPAD. **(B)** Total number of sips on yeast of flies expressing an IR against a non-neuronal gene CG9073[KK103240] and *CG12531* (IRKK). **(C)** Total number of sips on sucrose of flies expressing an IR a non-neuronal gene CG9073[KK103240] and *CG12531* (IRKK). **(D)** Total number of sips on yeast of flies expressing an additional hairpin (IRshmiR) targeting *CG12531*. **(E)** Total number of sips on sucrose of flies expressing an additional hairpin (IRshmiR) targeting *CG12531*. n=30-64 for all conditions; Mann-Whitney test or Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; $p>0.05$ ns, $p<0.05$*, $p<0.01$**, $p<0.0001$***.

amount of *CG12531* mRNA is indeed reduced in head extracts of knockdown flies **(Figure 6.10A and B)**. Altogether, and given the protein specific over-eating phenotype associated with knockdown of this gene, and the zeitgeist drink, I named the gene *beefeater*.

In summary, I show that the knockdown of *CG12531* via two RNAi's, non-overlapping in their target sequence in the gene of interest, produce the same yeast-feeding phenotype, strongly indicating that *CG12531* is required for the regulation of protein intake by the fly and not for sucrose.

As an additional method to ensure the LoF phenotype seen following RNAi mediated knockdown of *beefeater* is not due to non-specific effects of RNAi transgene expression, I also asked if flies mutant for *beefeater* would exhibit the same LoF phenotype. I used a *Minos*-mediated integration cassette (MiMIC) insertion line [25], where the MiMIC transgene is in the first coding intron of *beefeater,* MI13892 **(Figure 6.11A)**, in combination with a deficiency line, *Df(1)Exel6253*. The MiMIC transgene contains a splice acceptor site followed by three stop codons in three frames and a splice donor site, ensuring premature termination of translation. The mutant flies replicate the phenotype produced by the pan-neuronal knockdown, i.e., an increase in the number of sips on yeast **(Figure 6.11B)** and no change in sucrose feeding behaviour **(Figure 6.11C)**. As before, the downregulation of *beefeater* expression by the mutation was tested for, and only residual mRNA levels are present **(Figure 6.11D)**. The fact that the pan-neuronal knockdowns have the same phenotype as a mutant is in agreement with *beefeater* only having a function in the nervous system. Consistently, data available from Flybase

suggests that *beefeater* is only expressed in the nervous system of *Drosophila*.

To determine if *beefeater* might act in a sexually dimorphic manner I tested males to see if they show similar changes in yeast feeding to females. It is known that males exhibit a very different protein appetite to females; adult female flies are more sensitive to protein deprivation and switch their food preference to protein food much faster than males [4]. Remarkably, male *beefeater*$^{MiMIC/Df(1)Exel6253}$ flies showed no change in yeast feeding **(Figure 6.11E)**, suggesting this gene might be part of a mechanism only present in females.

To test if *beefeater* is also sufficient to regulate food choice in flies I designed a UAS construct to determine the effects of pan-neuronal over-expression of *beefeater*. Over-expression of this construct with *nSyb-Gal4* was sufficient to reduce the number of sips on yeast **(Figure 6.12A)**, whilst having no effect on sucrose in the amino acid deprived state **(Figure 6.12B)**. This GoF indicates that the levels of *beefeater* alone in the nervous system are sufficient to drive the changes seen in feeding behaviour. A quantification by qRT-PCR showed a small but significant increase in the amount of *beefeater* mRNA in heads of the GoF flies **(Figure 6.12C)**.

6.10

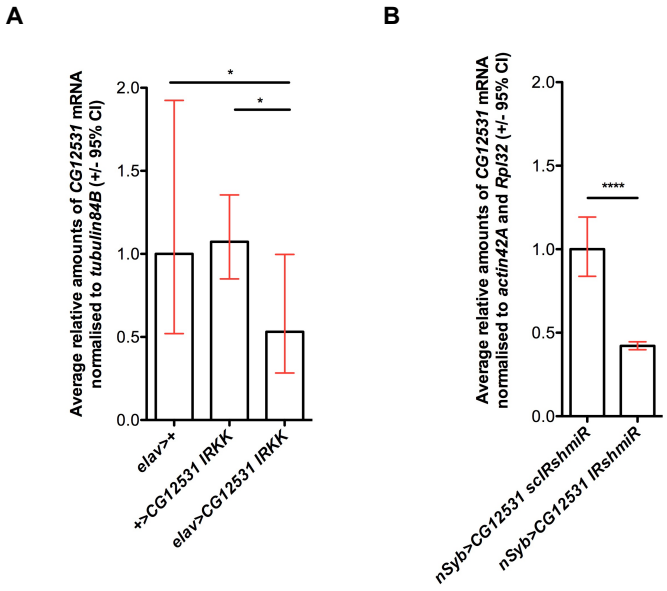


Figure 6.10. Pan-neuronal expression of both IRs targeting *CG12531* decrease *CG12531* mRNA levels. **(A)** *CG12531* transcript levels normalised to *tubulin84B* as obtained by qRT-PCR. RNA prepared from flies pan-neuronally expressing IRKK targeting *CG12531*. **(B)** *CG12531* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. RNA prepared from flies pan-neuronally expressing IRshmiR targeting *CG12531*. n=3; t-test or ANOVA; data shown as mean, error bars show 95% CI; $p<0.05$*, $p<0.0001$****.

6.11

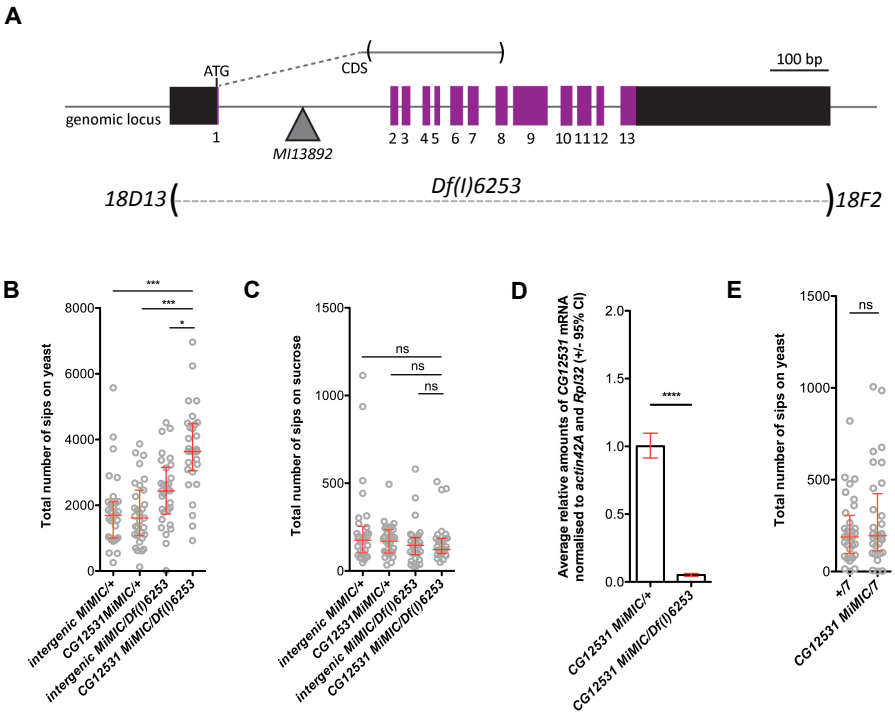


Figure 6.11. Flies mutant for *CG12531*/*beefeater* have increased yeast feeding, whilst sucrose feeding remains unchanged. **(A)** Gene diagram of *CG12531/beefeater*. UTRs in black, exons are in purple and numbered 1-13. Region deleted in mutants shown on coding DNA sequence (CDS) between brackets. Deficiency region deleted18D13-18F2. **(B and C)** Food intake was assessed using the flyPAD in female flies mutant for *CG12531/beefeater* (*CG12531^{MI13892}/Df(l)6253*) the control flies were the intergenic MiMIC line, *MI00606*. **(B)** Total number of sips on yeast and **(C)** total number of sips on sucrose. **(D)** *CG12531* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. n=3; t-test; data shown as mean, error bars show 95% CI; p<0.0001****. **(E)** Total number of sips on yeast in mutant male flies. **(B, C and E)** Mann-Whitney test or Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; p>0.05 ns, p<0.05*, p<0.0001***.

6.12

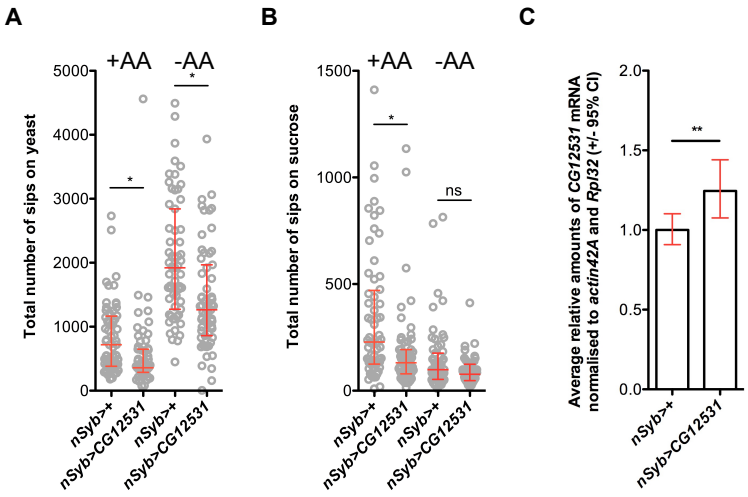


Figure 6.12. Flies over-expressing *CG12531*/*beefeater* in the nervous system have decreased yeast feeding. **(A and B)** Food intake was assessed using the flyPAD in female flies pan-neuronally over-expressing *CG12531*/*beefeater.* Flies were prefed the indicated diets, with (+AA) or without (-AA) amino acids. **(A)** Total number of sips on yeast and **(B)** total number of sips on sucrose. n=59-60 for all flyPAD conditions; Mann-Whitney test; data shown as median and IQR; p>0.05 ns, p<0.05*. **(C)** *CG12531*/*beefeater* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. n=3; t-test; data shown as mean, error bars show 95% CI; p<0.01**.

6.4.4. beefeater is not necessary for flies to respond to protein deprivation

Given that *beefeater* is required for the regulation of yeast feeding in amino acid fed female flies, I next asked whether *beefeater* might also have a role in the behavioural response of flies when protein or amino acids are removed from the diet. I first tested the ability of *beefeater* flies to respond to three days of protein deprivation (100mM sucrose). Both the control and *beefeater* flies exhibited a normal increase in the number of sips on yeast in the flyPAD **(Figure 6.13A)**, indicating that *beefeater* is not necessary for this homeostatic switch in feeding behaviour. Instead, perhaps *beefeater* is only necessary to maintain a set-point defining the base-line amount of protein rich food or amino acids a fly requires. Interestingly, whilst *beefeater* mutants exhibit an increased number of sips on yeast in the fed state, no such phenotype is apparent in the protein deprived condition, suggesting *beefeater* is not regulatory in the fly when protein levels are very low, or that protein deprivation already phenocopies loss of *beefeater*. Furthermore, when the flies were instead subject to amino acid deprivation, the number of sips on yeast are also increased compared to the fed (+AA) state, and there is no effect of loss of *beefeater* in amino acid deprived conditions, phenocopying protein deprivation **(Figure 6.13B).**

So how does *beefeater* regulate the protein/amino acid set-point in fed flies? We know it is by the modulation in the total number of sips on amino acid rich food, but we also know that the total number of sips is structured into a burst of sips with inter-burst-

6.13

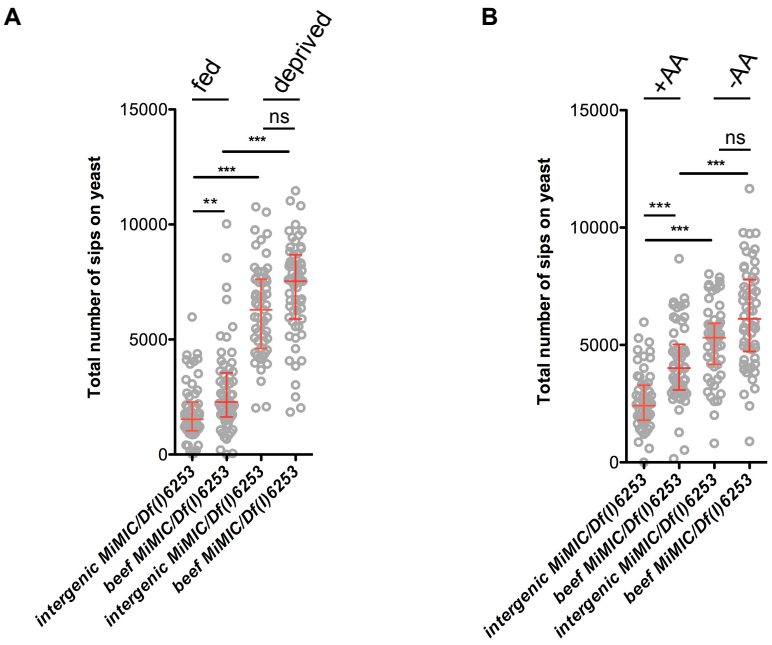


Figure 6.13. *CG12531*/*beefeater* is not necessary for flies to respond to protein/amino acid deprivation. (A) Total number of sips on yeast of flies mutant for *CG12531*/*beefeater* (*beef MiMIC/Df(l)6253*). Flies were prefed diets indicated at the top of the graph: yeast based food (fed) or sucrose (deprived). **(B)** Total number of sips on yeast of flies mutant for *CG12531*/*beefeater*. Flies were prefed diets indicated at the top of the graph: with amino acids (+AA) or without amino acids (-AA) for 3 days. n=55-61 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; p>0.05 ns, p>0.01 ** p<0.0001***.

6.14

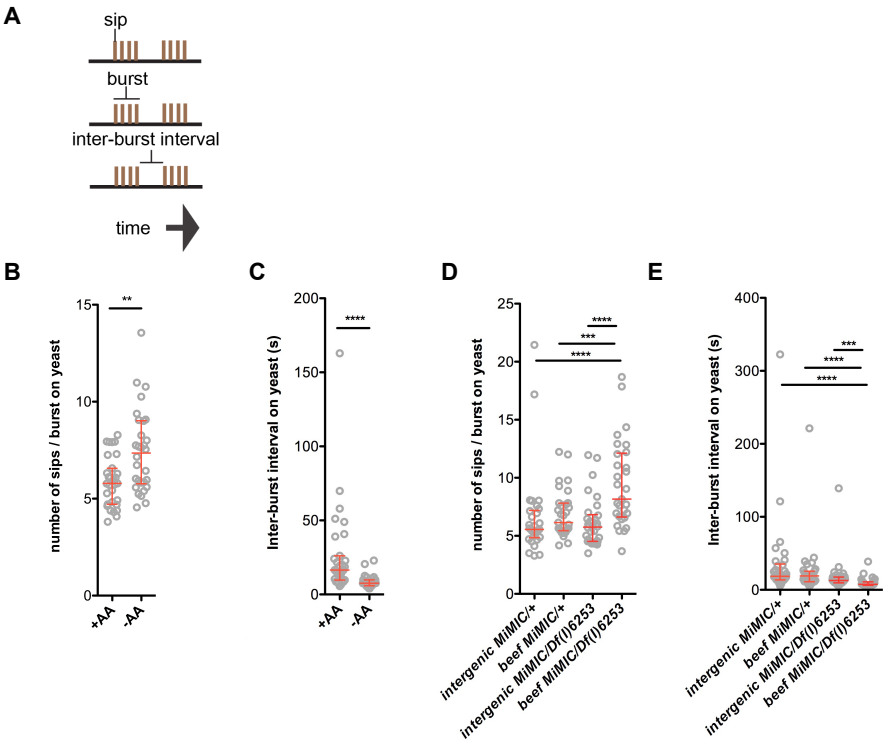


Figure 6.14. *CG12531*/*beefeater* changes feeding microstructure in the same way as amino acid deprivation. (A) Schematic showing the microstructure of feeding seen in the flyPAD setup. Sips (brown lines), can be grouped into bursts (groups of brown lines), with intervals in between (inter-burst intervals, spaces over time). **(B)** Average number of sips per burst per fly on yeast of w^{1118} flies prefed the diets indicated on the x-axis. **(C)** Average inter-burst interval on yeast of w^{1118} flies prefed the diets indicated on the x-axis. **(D)** Number of sips per burst on yeast of flies mutant for CG12531/*beefeater*. **(E)** Inter-burst interval on yeast of flies mutant for *CG12531/beefeater*. n=29-31 for all conditions; Mann-Whitney test or Kruskal-Wallis test followed by correction for multiple comparisons; data shown as median and IQR; p<0.05*, p<0.01**, p<0.0001***, p<0.0001****.

intervals **(Figure 6.14A)**. In the case of amino acid deprivation, where the total number of sips on yeast is increased, this is achieved by increasing the average number of sips per bust **(Figure 6.14B)** and decreasing the average interval between these bursts of sips **(Figure 6.14C)**. In the case of fed *beefeater* flies I also observe an increase in the average number of sips per burst and a decrease in the average interval between bursts of sips **(Figure 6.14D and E).** This similarity between the *beefeater* mutants and flies subject to amino acid deprivation may suggest a similar underlying neuronal mechanism controlling the total number of sips in both conditions.

6.4.5. beefeater is expressed in the nervous system

It has previously been published that *beefeater* is expressed in the heads of *Drosophila* [17] and Flybase expression data indicates that *beefeater* is almost exclusively expressed in the nervous system. In order to better characterise the expression pattern of *beefeater* I tested several commercially available antibodies against the human orthologue of *beefeater*, but none gave a specific signal in the fly brain (data not shown). To overcome this problem, I used the MiMIC line (*MI13892*) of *beefeater*. This transposon functions as a gene trap, with GFP as the reporter gene [25]. Using this line heterozygously to avoid any LoF effects, I found GFP to be broadly expressed in the brain, with no obvious expression in the glia **(Figure 6.15A)**. I also used a recombinase mediated cassette exchange (RCME) [26], to convert this line into a protein trap – tagging the endogenous Beefeater protein with a GFP containing construct

(EGFP::FlAsH::StrepII::3xFlag) 6 amino acids into the N-terminus of the coding region **(Figure 6.15B)**. The resulting fusion protein, beef::GFP::beef, is broadly expressed in the central brain **(Figure 6.15C)** and ventral nerve cord (VNC) **(Figure 6.15D).** There is also broad expression of beef::GFP::beef in the L3 larval brain **(Figure 6.15E)**. In contrast, I failed to see any expression in the peripheral nervous system, including neurons of the leg, wing, proboscis or antennae **(Figure 6.16)**. Together, these results indicate that the function of *beefeater* in regulating behaviour may be upstream of the peripheral neurons of olfaction and gustation, and instead localised in the central brain or perhaps the VNC.

6.4.6. *beefeater* is localised to the lysosome

I also sought to determine the subcellular expression of beefeater. For this, I first used the beef::GFP::beef line and saw small GFP positive puncta in the cytoplasm of neurons **(Figure 6.17A)**. However, when I tested this line (beef MiMIC rescue 3.7) for yeast feeding phenotypes in the flyPAD, I saw no reduction in the number of sips on yeast compared to the mutant flies, and a significant increase compared to the control flies **(Figure 6.17B**), suggesting the replacement of the MiMIC STOP codon with a GFP containing tag did not abolish the LoF. Importantly, the insertion of the GFP construct in the reverse orientation at the site of the MiMIC STOP codon (beef MiMIC rescue 3.8), and so not included as an extra exon, did partially rescue the behaviour, and the number of sips on yeast of the flies containing this reverse GFP construct was reduced compared to the mutant flies,

6.15

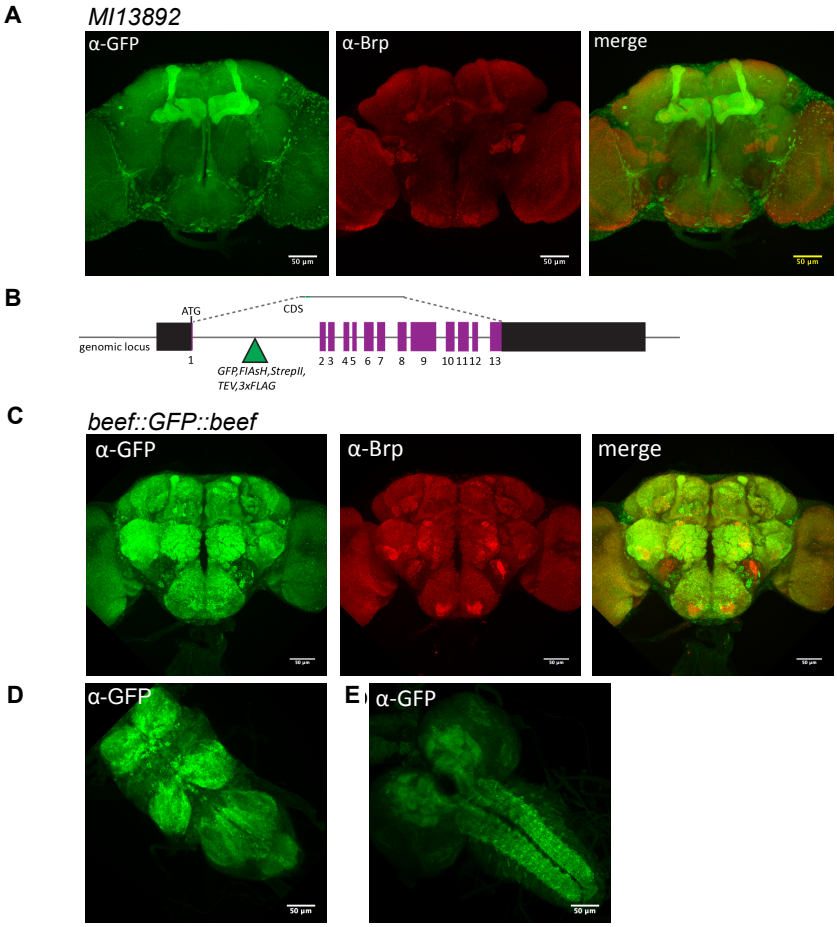


Figure 6.15. *CG12531*/*beefeater* is broadly expressed in the nervous system. **(A)** Brains dissected from heterozygous *MI13892* adult female flies. Co-staining with anti-Brp (NC82). **(B)** Gene diagram of *CG12531*/*beefeater*. UTRs in black, exons are in purple and numbered 1-13. Site of Recombinase Mediated Cassette Exchange (RCME) and tag is marked with green triangle. **(C)** beef::GFP::beef expression in adult female brains. Co-staining with anti-Brp (NC82). **(D)** beef::GFP::beef expression in adult female ventral nerve cord (VNC). **(E)** beef::GFP::beef expression in L3 female brains. Images presented as maximum intensity projections. Scale bars 50μm.

6.16

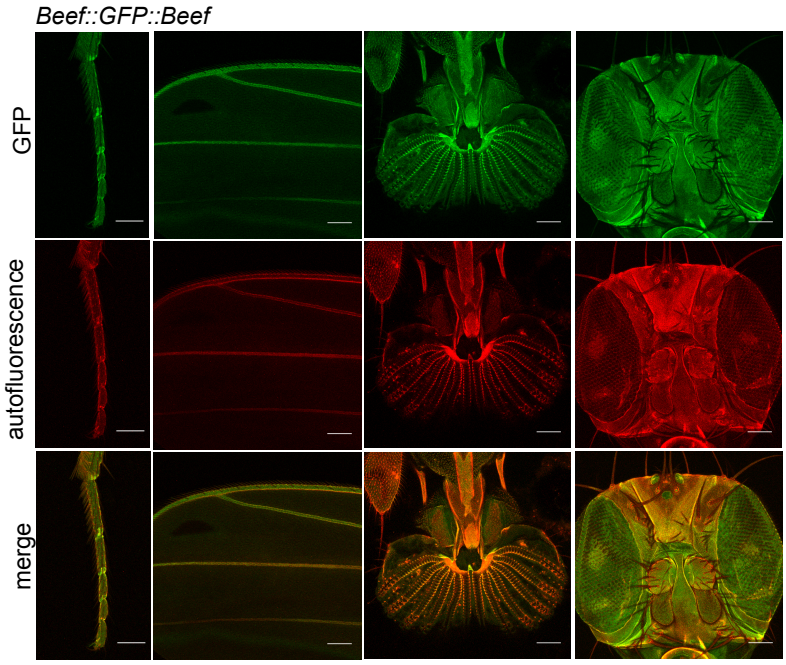


Figure 6.16. *CG12531/beefeater* has no apparent expression in sensory neurons. Beef::GFP::Beef expression in adult female legs (scale bar 100μm), wings (scale bar 100μm), proboscis scale bar 50μm) and antennae (scale bar 100μm). Images presented as maximum intensity projections.

although not to the levels of the control flies **(Figure 6.17C)**. This suggests the insertion of this GFP containing construct as an extra exon in the protein may lead to the misfolding and loss of function of the native protein. The partial rescue of the reverse orientation GFP construct suggests that the intronic region of *beefeater* may be important for transcriptional control of this gene and a LoF phenotype. As such, the beef::GFP::beef construct could not be used to assess the subcellular localisation of Beefeater. Instead, I used a UAS-tagged version of Beefeater with an mCherry tag at the C-terminus. I used the pan-neuronal driver *nSyb-Gal4* to over-express this tagged version of Beefeater **(Figure 6.18A)**. Flies showed a reduction in the number of sips on yeast **(Figure 6.18B)**, as seen previously with the over-expression of the untagged version of Beefeater **(Figure 6.12A)**, indicating that this tagged version is functional.

I then compared the subcellular localisation of this construct to that of CD8::GFP **(Figure 6.18C)** and Lamp1::GFP **(Figure 6.18D)**. Colocalisation is evident only with the lysosomal marker, suggesting Beefeater localises to the lysosome and not the plasma membrane. This localisation is conserved in the in humans, where the orthologue, SLC7A14, is found in human cell lines to localise at the lysosome [27]. This data indicates Beefeater may not simply be playing a role in the transport of amino acids into the neurons, but could be acting on the regulation of amino acid levels inside the neuron. Lysosomes are a sink for amino acids, as they are the end point of a key cellular degradation pathway, autophagy, where proteins are broken down to amino acids to be used again by the cell. Interestingly, lysosomes are also a site of the highly conserved nutrient sensing mTOR

pathway activity. Furthermore, they are capable of generating signalling molecules in response to certain inputs that can travel to he nucleus to activate transcriptional responses or to distant tissues to activate global homeostatic responses [28]. As such, beefeater is perfectly localised to play a central role in the amino acid homeostasis of the neuron.

6.4.7. Dissecting the cellular pathways regulated by *beefeater*

The mechanistic Target Of Rapamycin (mTOR) is a highly conserved nutrient sensing pathway, found in many cells, coordinating growth with nutrient availability [29]. It has been shown in mammalian cell lines that amino acids induce the movement of mTORC1 to lysosomal membranes, where together with the proteins of the Ragulator and RagGTPases, they activate mTORC1 [30] **(Figure 2.1)**. However, the molecular nature of amino acid sensing mechanisms has remained elusive. Several members of the SLCs predicted to transport amino acids at the plasma membrane have been shown to regulate mTOR activity [31]. Furthermore, SLC38A9 was found to act at lysosomes as a component of the lysosomal amino acid sensing machinery controlling mTOR [12,13]. In addition, mTOR pathway activity has been implicated as important in the regulation of feeding behaviour [4,22,32]. Given this I asked whether *beefeater* could be playing a similar role at the lysosome in controlling mTOR pathway activity in the neurons of *Drosophila*. If *beefeater* is

6.17

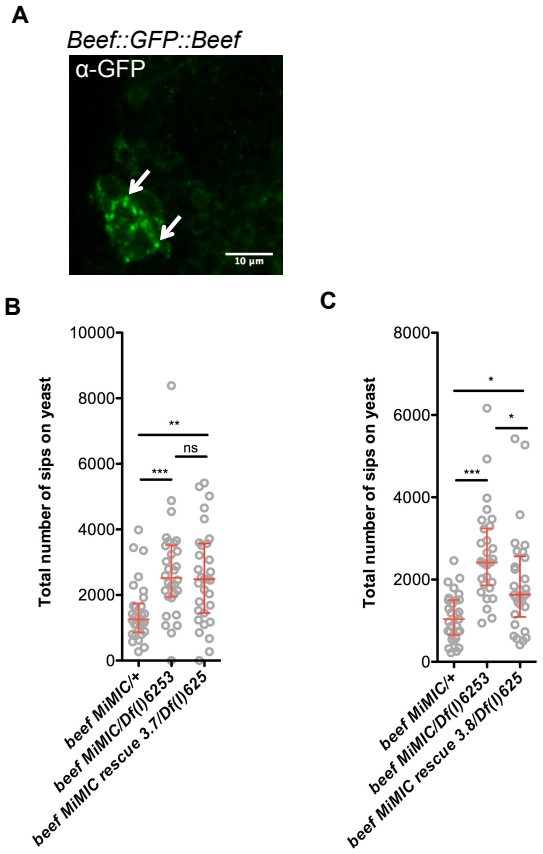


Figure 6.17. Beef::GFP::Beef is a non-functional protein. **(A)** Beef::GFP::Beef expression in adult neurons. Image presented as selected z-slice. Arrows indicate puncta in cytoplasm of neuronal cell bodies. Scale bar 10µm. **(B)** Total number of sips on yeast of flies in which the original MiMIC stop cassette has been replaced with a GFP containing construct to tag the endogenous protein (MiMIC 3.7 rescue). **(C)** Total number of sips on yeast of flies in which the original MiMIC stop cassette has been replaced with a GFP containing construct in the reverse orientation (MiMIC 3.8 rescue). n=29-31 for all conditions; Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; $p>0.05$ ns, $p<0.05$*, $p<0.001$***.

6.18

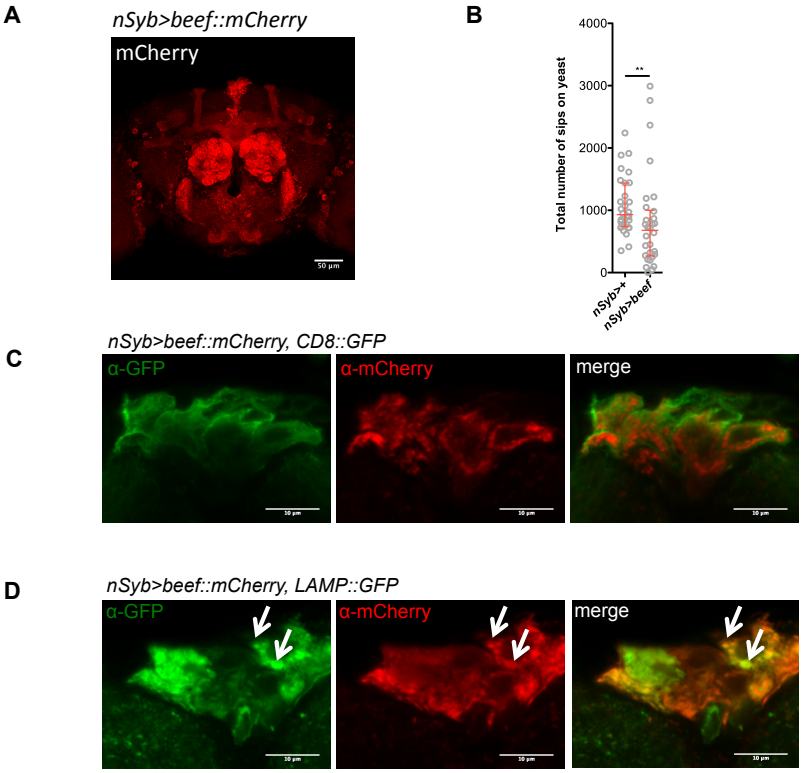


Figure 6.18. beef::mCherry is functional, and localises to the lysosome. (A) Pan-neuronal (*nSyb-Gal4*) driven expression of *UAS-beef::mCherry* in adult female brains. Image presented as maximum intensity projection. **(B)** Total number of sips on yeast of flies in which beef::mCherry is pan-neuronally over-expressed. n=30; Mann-Whitney test; data shown as median and IQR; p<0.01**. **(C)** Pan-neuronal (*nSyb-Gal4*) driven expression of *UAS-beef::mCherry* in adult female neurons with CD8::GFP as a marker of cell membranes. **(D)** Pan-neuronal (*nSyb-Gal4*) driven expression of *UAS-beef::mCherry* in adult female neurons with LAMP::GFP as a marker of lysosomes. Arrows mark sites of colocalisation. Images presented as selected z-slices. Scale bars 10μm.

capable of regulating mTOR pathway activity this could represent a mechanism though which food choice behaviour is regulated.

If *beefeater* lies upstream of mTOR signalling at the lysosome, perhaps its absence would inhibit the activation of mTOR, resulting in similar cellular responses as those seen after dietary amino acid deprivation. Having analysed some of the cellular responses to changing levels of mTOR activity in chapter 4, I used these same assays as readouts for mTOR activity in *beefeater* mutant flies. I looked first at whether *beefeater* may influence the phosphorylation of dS6K, I compared the levels of the phosphorylated protein in heads taken from fed *beefeater* mutant flies with those of *beefeater* heterozygous flies **(Figure 6.19A)**, but saw no consistent decrease in the dS6K-P signal **(Figure 6.19B)**. Furthermore, when assessing the levels of *unk* transcript there was no change in the levels when comparing these genotypes **(Figure 6.19C)**. In the assays quantifying autophagic activity, whether by the cytoplasmic to membrane bound transition of Atg8a, or by the levels of *ref(2)P* transcript, no changes were seen **(Figure 6.19D,E and F).** This data is in stark contrast with that observed following dietary amino acid deprivation. The fact that *beefeater* LoF has no quantifiable effects on the activity of the mTOR pathway or the modulation of pathways downstream of mTOR indicates that *beefeater* is not upstream of mTOR.

An alternative hypothesis is that *beefeater* is part of the mTOR pathway, but lies downstream of the effects so far quantified. To test this hypothesis, I first tested if genetic manipulation of the mTOR pathway activity would phenocopy that of the *beefeater* mutant – if we see a change in the number of sips on yeast, but

nothing in the number of sips on sucrose, they phenocopy each other and this would suggest involvement in the same pathway. I over-expressed the mTOR inhibitors *TSC1* and *TSC2* pan-neuronally, and then used the flyPAD to quantify changes in feeding behaviour. When these flies were kept on food with amino acids no significant changes were seen in either yeast **(Figure 6.20A)** or sucrose feeding **(Figure 6.20B)**. When the same flies were prefed food containing no amino acids, I still observed no change in yeast sips **(Figure 6.20C)**, but a small reduction in the number of sips on sucrose became apparent **(Figure 6.20D)**. Interestingly, it has previously been shown, and quantified in a 2-choice colourimetric assay [4], that the over-expression in the nervous system of the inhibitors TSC1 and TSC2 did affect yeast preference in male flies that had been deprived of dietary protein. The lack of any effect in this experiment could be assay specific – the colourimetric assay is ratiometric, and so the ratio between yeast and sucrose eaten contributes to the preference. Or the result could be context specific, in that protein deprivation drive different pathways to amino acid deprivation – for example, it is well established that the *TSC1,TSC2* complex inhibits mTOR activity, it has also been shown that this complex is not always necessary for the response of mTOR to amino acids [33]. Finally, the phenotype could be specific to males. I therefore induced the expression of these mTOR inhibitors in neurons of flies over-expressing *beefeater*, to test for a genetic interaction. The expression of both *UAS-TSC1* and *UAS-TSC2* in a *beefeater* GoF background fails to modify the reduction in the number of yeast sips that is produced by *beefeater* over-expression **(Figure 6.21A)**. Taken together, the lack of similarity between the *beefeater* LoF

phenotype and mTOR manipulations, and the lack of any genetic interaction between *beefeater* and mTOR manipulations suggests that further genetic manipulations of mTOR pathway activity would need to be done to fully address this issue of the interaction between mTOR and *beefeater*.

In an attempt to characterise a role for *beefeater* in other cellular pathways, I explored its involvement in the pathways controlling insulin secretion. In the brain of the fly, the insulin-producing cells (IPCs) are functionally analogous to the β cells of mammals [34]. The IPCs produce 4 insulin-like peptides (DILP1, 2, 3, and 5). In *Drosophila* larvae it has been shown that Dilp2 and Dilp5 show a strong accumulation in the brain insulin producing cells (IPCs) in response to dietary protein/amino acid deprivation and that this is associated with decreased transcript levels of both peptides [35]. If *beefeater* regulates the release of insulin-like peptides in a manner similar to proteins or amino acids, we might expect in the mutant to see both increased levels of Dilp2 and Dilp5 peptides in IPCs and decreased levels of *Dilp2* and *Dilp5* transcript. I took amino acid fed mutant flies and quantified the amount of *Dilp2* and *Dilp5* transcript in head extracts. However, no significant changes were seen in the mutant flies compared to the controls **(Figure 6.22A and B)**, indicating that *beefeater* is not involved in the pathways controlling insulin release.

6.4.8. Finding the neurons in which *beefeater* acts

The finding that *beefeater* is expressed broadly throughout the nervous system raises two possible models for where *beefeater*

6.22

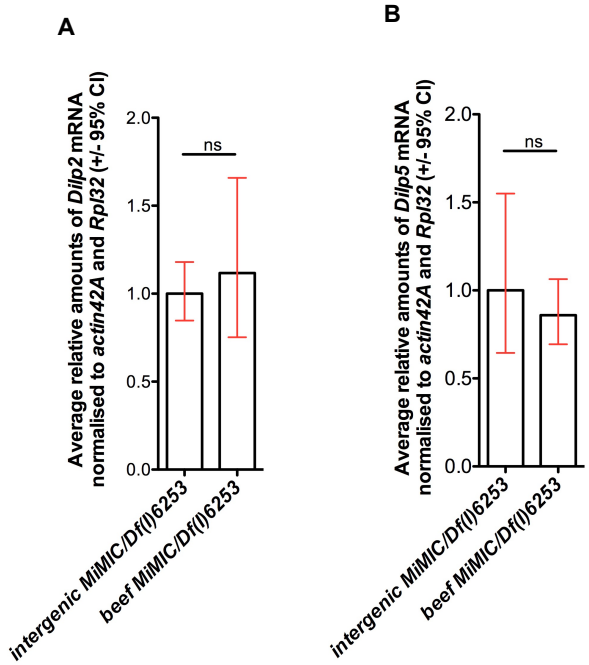


Figure 6.22. Insulin signalling is not affected in *CG12531/beefeater* mutants. **(A)** *Dilp2* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. Female head extracts of CG12531/*beefeater* mutants flies (*beefMiMIC/ Df(l)6253*) and controls. **(B)** *Dilp5* transcript levels normalised to *actin42A* and *Rpl32* as obtained by qRT-PCR. Female head extracts of CG12531/*beefeater* mutants flies (*beefMiMIC/Df(l)6253*) and controls. n=3; significance tested using t-test; data shown as mean, error bars show 95% CI; p>0.05 ns.

is required in the nervous system to mediate food choice. Either, *beefeater* is required in all the neurons it is expressed in to regulate food choice behaviour, or, *beefeater* it is only required in a subset of these neurons in which is it expressed to regulate food choice behaviour, and in the neurons not necessary for behaviour *beefeater* fulfills an independent function. In order to test the second hypotheses, I took an assortment of Gal4 driver lines that are made from the enhancer regions of both neurotransmitters and neuropeptides and therefore target subpopulations of central neurons, to ask if the LoF of *beefeater* in any of these smaller sets of neurons is sufficient to modulate the flies feeding behaviour in the same way as the pan-neuronal manipulation. First, I induced the expression of the original RNAi line identified in the screen with the assortment of Gal4s. I found no Gal4 driver where the knockdown of *beefeater* was sufficient to increase the number of sips on yeast **(Figure 6.23A)**. Interestingly I only found a selection of neurons in which *beefeater* knockdown appears to give the reverse phenotype, that is, of reducing the number of sips on yeast. I therefore re-screened these Gal4 lines with the second RNAi – its short sequence makes it less likely to have off-target effects, and the 'scrambled' hairpin is a more precise control. With this setup, I identified three Gal4 lines with which the LoF of *beefeater* increased the number of sips on yeast, *dimmed-Gal4*, *GAD-Gal4* and *Dilp2-Gal4* **(Figure 6.23B)**, all phenocopying the pan-neuronal knockdown phenotype of *beefeater*. Interestingly, *Ddc-Gal4*, a driver labelling some serotoninergic and octopaminergic neurons, produced a decrease in the total number of sips on yeast.

6.23

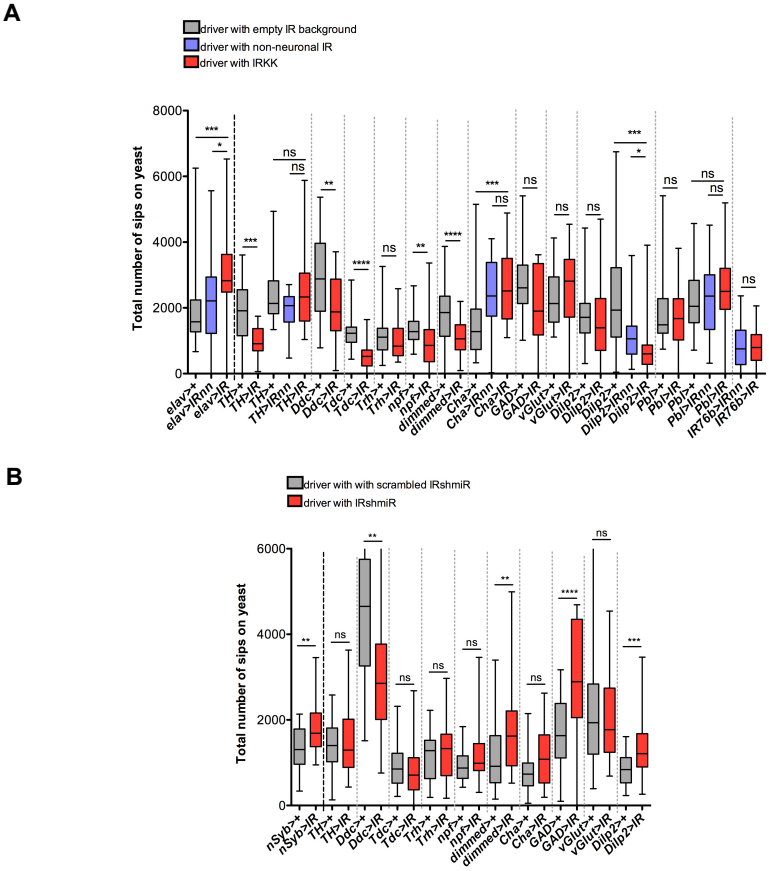


Figure 6.23. Finding the neurons in which *CG12531/beefeater* acts to control feeding behaviour. (A) A series of Gal4 lines were chosen to express an IR against CG12531/*beefeater* (IRKK). Food intake was assessed using the flyPAD. Total number of sips on yeast was quantified. **(B)** The same set of Gal4 lines were also tested with an additional RNAi line (IRshmiR). n=16-32 for all conditions. Mann-Whitney test; boxes show median and IQR, and whiskers show minimum/ maximum values; p>0.05 ns, p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

6.24

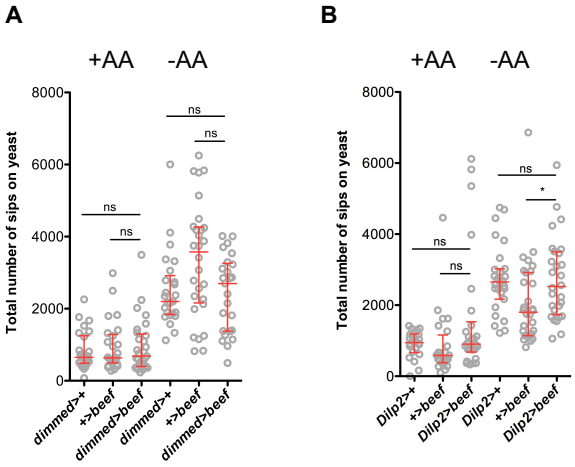


Figure 6.24. Over-expression of *CG12531/beefeater* in dimmed- or Dilp2-neurons has no effect on feeding behaviour. (A) Total number of sips on yeast of *Dimmed>beef* flies. Flies were prefed a diet either with (+AA) or without (-AA) amino acids, as indicated on the graph. **(B)** Total number of sips on yeast of *Dilp2>beef* flies. Flies were prefed a diet either with (+AA) or without (-AA) amino acids, as indicated on the graph. n=25-30 for all conditions. Kruskal-Wallis test followed by Dunns multiple comparisons; data shown as median and IQR; $p>0.05$ ns, $p<0.05$*.

Dimmed-Gal4 and *Dilp2-Gal4* are known to overlap in the Dilp2 neurons [36], and represent much smaller populations of neurons in the fly brain than *GAD-Gal4*, so I chose to focus on these. The major concern here is that I do not see the same phenotype with the original KK RNAi line. To overcome this, I tested the GoF of *beefeater* with the same Gal4 drivers to ask if this is sufficient to reverse the phenotype. However, the *beefeater* over-expressing flies showed no reduction in the number of sips on yeast with *dimmed-Gal4* **(Figure 6.24A)**, or with *Dilp2-Gal4* **(Figure 6.24B)**. Whilst it is possible that these drivers are not strong enough to induce the Beefeater levels required to elicit the decreased feeding on yeast, it is also possible that the phenotype following expression of the shmiR transgene is a non-specific phenotype. Therefore, it is not possible at this moment to either confirm or exclude the possibility that these neuronal subpopulations are a specific site for *beefeater* function.

6.5. Discussion

How an animal updates its behaviour in a coordinated fashion, and according to its physiological state, is a complex problem. The identification of *beefeater* as a neuronal gene driving protein appetite represents a major advance in our understanding of this problem. *Beefeater* is a member of the SLC7 family of amino acid transporters [37]. A family that also includes the transporter *slimfast*, which plays a key role in the fat body as a nutrient sensor controlling growth [11], *genderblind*, which modulates

courtship behaviour [38], and *minidiscs,* which senses extracellular leucine to regulate insulin release [39].

Beefeater appears to only be expressed in the nervous system of the fly, and within the nervous system it localises to the lysosomes. These results suggest *beefeater* may be regulating or detecting, depending on whether it is acting as a transporter or transceptor, lysosomal-cytosolic amino acid levels in neurons to set a base line of intracellular 'amino acid availability'. In *beefeater* mutants, flies could lose access to this lysosomal pool of amino acids, leading to increased yeast feeding in these flies. The fact that the increased yeast feeding phenocopies changes seen in feeding microstructure following amino acid deprivation suggests *beefeater* may be able to harness the same neuronal feeding pathways as amino acids to regulate feeding. However, *beefeater* is not necessary for flies to shift their behaviour following amino acid deprivation, suggesting that although *beefeater* can modulate the feeding pathways, it is not the only input.

When considering what cellular pathways might be controlled by *beefeater* activity, there are two candidates – mTOR and GCN2. Both of these pathways are highly conserved nutrient sensing pathways that have been described as regulating feeding behaviour. *Slimfast*, an amino acid transporter in the same family as *beefeater* has previously been described as modulating the GCN2 pathway in neurons of *Drosophila* larvae [3], and the mTOR pathway in the fat body, again of *Drosophila* larvae [11]. Whilst previous data collected in the lab does not strongly support a role for GCN2 in the selection and consumption of protein rich food (data not shown), there is evidence to suggest

the involvement of neuronal mTOR signalling in the regulation of food choice [4]. Furthermore, it has been shown that amino acid transporters can interact with the mTOR pathway at the lysosomal membrane [12, 13]. Goberdhan and colleagues even coined the term 'nutrisome' for this amino acid sensing complex at the lysosome [21]. One hypothesis is that *beefeater* may function to modulate mTOR signalling at the lysosome, and that in turn affects food choice behaviour. However, I found no evidence that supports a role for *beefeater* in regulating activity in the mTOR pathway. Furthermore, I see no genetic interaction between the *beefeater* GoF phenotype and expression of the mTOR inhibitors TSC1 and TSC2. From this data, I cannot state any role for *beefeater* in the regulation of neuronal mTOR pathway activity.

The localisation of beefeater at the lysosome suggests the lysosome itself may play a key role in determining the nutrient status of the neuron and in the regulation of feeding behaviour. There is evidence that the lysosome is capable of generating signalling molecules that can travel to the nucleus of the cell to activate transcriptional responses or to distant tissues to activate global homeostatic responses [28]. Perhaps *beefeater* could be regulating these functions of the lysosome.

Finally, there is a human orthologue of *beefeater*, SLC7A14 (44.57 sequence identity when the full-length proteins are compared, Clustal, but not so close to other SLC7 family members, SLC7A9 and SLC7A11, 20.23% and 23.7% PI) which has also been described as transporting amino acids into the lysosome in the nervous system [40]. Whilst SLC7A14 has been

studied in the context of retinitis pigmentosa [27], no one has yet examined if these knock out animals have any changes in feeding behaviour. Or, even further, to test if any polymorphisms/mutations exist in the human forms of the protein that are linked with eating or metabolic disorders. If this were found to be the case, *beefeater* might represent an interesting new drug target in the future in the treatment of metabolic disorders.

6.6. References

1. Masse, N. Y., Turner, G. C. & Jefferis, G. S. X. E. Olfactory information processing in Drosophila. *Curr. Biol.* **19,** R700-13 (2009).

2. Yarmolinsky, D. a, Zuker, C. S. & Ryba, N. J. P. Common sense about taste: from mammals to insects. *Cell* **139,** 234–44 (2009).

3. Bjordal, M. *et al.* Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in Drosophila. *Cell* **156,** 510–21 (2013).

4. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

5. Fromentin, G. *et al.* Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutr. Res. Rev.* **25,** 29–39 (2012).

6. Pool, A. H. & Scott, K. Feeding regulation in Drosophila. *Curr. Opin. Neurobiol.* **29,** 57–63 (2014).

7. Herbert, S. L. & Ribeiro, C. Nutrition: rejection is the fly's protection. *Curr. Biol.* **24,** R278-80 (2014).

8. Karnani, M. M. *et al.* Activation of central orexin/hypocretin

neurons by dietary amino acids. *Neuron* **72,** 616–29 (2011).

9. Leitão-Gonçalves, R. *et al.* Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLOS Biol.* **15,** e2000862 (2017).

10. Goberdhan, D. C. I., Meredith, D., Boyd, C. a R. & Wilson, C. PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids. *Development* **132,** 2365–2375 (2005).

11. Colombani, J. *et al.* A nutrient sensor mechanism controls Drosophila growth. *Cell* **114,** 739–749 (2003).

12. Rebsamen, M. *et al.* SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature* **9,** (2015).

13. Wang, S. & Tsun, Z.-Y. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science (80-.).* **347,** (2015).

14. Piper, M. D. W. *et al.* Matching Dietary Amino Acid Balance to the In Silico- Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan. *Cell Metab.* **25,** 610–621 (2017).

15. Itskov, P. M. *et al.* Automated monitoring and quantitative analysis of feeding behavior in Drosophila. *Nat. Commun.* 4560 (2014).

16. Boudko, D. Y. Molecular basis of essential amino acid transport from studies of insect nutrient amino acid transporters of the SLC6 family (NAT-SLC6). *J. Insect Physiol.* **58,** 433–449 (2012).

17. Romero-Calderón, R. *et al.* A screen for neurotransmitter transporters expressed in the visual system of Drosophila melanogaster identifies three novel genes. *Dev. Neurobiol.* **67,** 550–569 (2007).

18. Sundberg, B. E. *et al.* The evolutionary history and tissue mapping of amino acid transporters belonging to solute carrier families SLC32, SLC36, and SLC38. *J. Mol. Neurosci.* **35,** 179–93 (2008).

19. Green, E. W., Fedele, G., Giorgini, F. & Kyriacou, C. P. A Drosophila RNAi collection is subject to dominant phenotypic effects. *Nat. Methods* **11,** 222–3 (2014).

20. Piper, M. D. W. *et al.* A holidic medium for Drosophila melanogaster. *Nat. Methods* **11,** 100–5 (2014).

21. Ögmundsdóttir, M. H. *et al.* Proton-assisted amino acid transporter PAT1 complexes with Rag GTPases and activates TORC1 on late endosomal and lysosomal membranes. *PLoS One* **7,** e36616 (2012).

22. Vargas, M. A., Luo, N., Yamaguchi, A. & Kapahi, P. A Role for S6 Kinase and Serotonin in Postmating Dietary Switch and Balance of Nutrients in D. melanogaster. *Curr. Biol.* **20,** 1006–1011 (2010).

23. Haley, B., Hendrix, D., Trang, V. & Levine, M. A simplified miRNA-based gene silencing method for Drosophila melanogaster. *Dev. Biol.* **321,** 482–490 (2009).

24. Lin, W. *et al.* The SLC36 transporter Pathetic is required for extreme dendrite growth in Drosophila sensory neurons. *genes Dev.* 1120–1135 (2015). doi:10.1101/gad.259119.115.and

25. Venken, K. J. T. *et al.* MiMIC: a highly versatile transposon insertion resource for engineering Drosophila melanogaster genes. *Nat. Methods* **8,** 737–743 (2011).

26. Nagarkar-jaiswal, S. *et al.* A library of MiMICs allows tagging of genes and reversible , spatial and temporal knockdown of proteins in Drosophila. *Elife* 1–28 (2015). doi:10.7554/eLife.05338

27. Jin, Z. *et al.* SLC7A14 linked to autosomal recessive retinitis pigmentosa. *Nat. Commun.* 1–9 (2014). doi:10.1038/ncomms4517

28. Mony, V. K., Benjamin, S. & O'Rourke, E. J. A lysosome-centered view of nutrient homeostasis. *Autophagy* **12,** 619–631 (2016).

29. Jewell, J. L. *et al.* Differential regulation of mTORC1 by leucine and glutamine. *Science* (2015). doi:10.1126/science.1259472

30. Sancak, Y. *et al.* Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141,** 290–303 (2010).

31. Nicklin, P. *et al.* Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell* **136,** 521–534 (2009).

32. Cota, D. Hypothalamic mTOR Signaling Regulates Food Intake. *Sci. (New York, NY)* **312,** 927–930 (2006).

33. Smith, E. M., Finn, S. G., Tee, A. R., Brownei, G. J. & Proud, C. G. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J. Biol. Chem.* **280,** 18717–18727 (2005).

34. Géminard, C. *et al.* Control of metabolism and growth through insulin-like peptides in Drosophila. *Diabetes* **55,** 5–8 (2006).

35. Géminard, C., Rulifson, E. & Léopold, P. Remote Control of Insulin Secretion by Fat Cells in Drosophila. *Cell Metab.* **10,** 199–207 (2009).

36. Hewes, R. S., Park, D., Gauthier, S. a, Schaefer, A. M. & Taghert, P. H. The bHLH protein Dimmed controls neuroendocrine cell differentiation in Drosophila. *Development* **130,** 1771–1781 (2003).

37. Fotiadis, D., Kanai, Y. & Palacín, M. The SLC3 and SLC7 families of amino acid transporters q. *Mol. Aspects Med.* **34,** 139–158 (2013).

38. Grosjean, Y., Grillet, M., Augustin, H., Ferveur, J.-F. & Featherstone, D. E. A glial amino-acid transporter controls synapse strength and courtship in Drosophila. *Nat. Neurosci.* **11,** 54–61 (2008).

39. Maniere, G., Ziegler, A. B., Geillon, F., Featherstone, D. E. & Grosjean, Y. Direct Sensing of Nutrients via a LAT1-like Transporter in Drosophila Insulin-Producing Cells. *Cell Rep.* **17,** 137–148 (2016).

40. Jaenecke, I. *et al.* A chimera carrying the functional domain of the orphan protein SLC7A14 in the backbone of

SLC7A2 mediates trans-stimulated arginine transport. *J. Biol. Chem.* **287,** 30853–30860 (2012).

41. Sun J, Liu C, Bai X, Li X, Li J, Zhang Z, Zhang Y, Guo J, Li Y. Drosophila FIT is a protein-specific satiety hormone essential for feeding control. *Nat. Comm.* 8-14161 (2017).

6.7. Acknowledgements

The Vienna Drosophila resource center (VDRC) for RNAi stocks

Dr. Zita Carvalho-Santos for help with bioinformatics analysis on predicted amino acid transporters

Dr. Ana Paula Elias for cloning the *beefeater::mCherry* fusion construct

The Champalimaud Fly Platform for all embryo injections

Dr. Pavel Itskov for help with MatLab in processing of FlyPAD data.

Chapter 7

7.1. Discussion and conclusions

Understanding how the nervous system responds to and regulates amino acid homeostasis is key in unravelling how an animal is able to maintain a healthy life in terms of growth, reproduction and aging. Dietary protein or amino acids are a particularly important component of an animal's diet and many of the amino acids required for cellular processes, such as protein biosynthesis, neurotransmission and antioxidant defence, can only be obtained through a food source [1]. As such, an animal must have cellular mechanisms in place capable of detecting internal protein or amino acid availability; of comparing these amino acid levels with the current state; and, finally, of modifying the feeding behaviour of the animal according to either the ingestion or the rejection of a particular food source. The role of amino acid sensing by the nervous system is essential in this process as it provides a mechanism to efficiently link the behavioural output of an organism to the amino acid status.

The work presented in this thesis first demonstrates that the ratio of amino acids in a protein source is crucial in determining the quality of that protein for the animal. I then go on to present evidence that the nervous system can respond to dietary protein and amino acid deprivation by down-regulating activity in the nutrient sensitive mTOR pathway and that of downstream mTOR regulated pathways. I also present evidence that this could be in response to fluctuating levels of amino acids within the nervous system. Finally, I was able to identify a novel mechanism

whereby the nervous system is able to regulate amino acid homeostasis of the animal through neuronal *beefeater* mediated regulation of food choice.

7.1.1. Exome-matching represents a novel framework to define the quality of a protein source

Throughout nature there exists many examples of animals balancing both the quantity and quality of protein in their diet to optimise life history traits. Namely, it has been shown that flies can choose a diet with a protein:carbohydrate ratio that optimises their lifetime egg production [2]. However, the rules dictating the requirements for quantity and quality of protein of an animal remain largely unknown. In this study, I describe a set of experiments and respective results supporting the thesis that a ratio of amino acids matching that of *Drosophila's* protein coding regions within the genome – the exome – may well provide a framework for defining a balanced or high quality protein diet for a fly. I show that an exome-matched diet is more satiating to the fly, which minimises the quantities of nutrients ingested. Interestingly, I have also shown that the exome-matched diet is more appetitative to flies, implying that the animal's failure to eat more from this diet is due to its satiating effects. In brief, the higher the quality of the protein composition, the lower the quantity an animal needs to eat of it as part of a balanced diet. The quantity of protein an animal eats is known to have a huge impact on the life history traits of that animal. A diet with high amounts of protein is associated with high reproduction and shorter lifespans, whereas a diet with low amounts of protein is associated with increased lifespans, but lower reproduction rates.

Remarkably, when flies are kept on an exome matched diet they lay more eggs with no cost to the animal's lifespan [3], indicating that a diet of high quality protein, i.e., where intake is reduced, can optimise reproduction and lifespan.

In the wild, flies feed on rotting fruit and the yeasts that grow on the decomposing surface. So how can flies hope to obtain a diet matching the amino acid ratio of their exome? Whilst it is possible that the exome-matched diet may not be ecologically relevant, an alternative hypothesis is that ingestion of different yeasts with different amino acid ratios, some of which have been shown to vary with environmental conditions [4] may add enough variety to the flies' diet for a match to their exome. Alternatively, cannibalism may also play a role in this optimisation [5]. *Drosophila* larvae have been shown to resort to cannibalism under crowded conditions and adult flies may consume dead larvae or dead adults if found around a food source.

In future experiments, it will be important to determine both the nutrient sensing mechanisms that drive the satiating effects of the exome-matched diet and determine its appetitiveness. It is known that the GCN2 pathway plays a role in a few dopamine neurons of the nervous system in mediating the rejection of imbalanced foods in *Drosophila* larvae [6]. This mechanism could also drive the reduction in appetitiveness of the mismatched diets compared to exome-matched diets.

In addition, there is potential relevance in this finding for human health, and future studies will require one to determine if the information encoded in the human exome could also be used to

design diets of higher protein quality to improve human lifespan whilst reducing overall intake.

7.1.2. Neuronal mTOR activity and mTOR regulated pathways respond to changes in dietary amino acids

As mentioned, a balanced diet is one containing all the macronutrients and micronutrients necessary for optimal growth, reproduction and lifespan. When a macro- or micronutrient is missing from the diet or available in insufficient or excess quantities, the animal needs to modify its feeding behaviour in order to consume more or less of this nutrient, and return the levels of this nutrient to the animals original set-point. This is termed homeostatic feeding, and it provides a mechanism to help animals maintain a balanced diet. For example, it has been shown that *Drosophila* increase their feeding on yeast, a protein rich food source, following a period of only having access to a protein deficient diet [7]. Furthermore, animals consume less of a protein-containing food if this food is deficient in one or more amino acids [8, 9]. The cellular mechanisms used in the nervous system that underlie these behavioural responses are beginning to be explored, and it is known that both the mechanistic Target Of Rapamycin (mTOR) pathway and the General Control Nonderepressing 2 (GCN2) pathway both play roles [10]. However, the exact nature of how the nervous system uses these pathways to sense availability of amino acids, integrate this information and convert it into a behavioural modification is uncertain.

Earlier observations showed that mTOR activity was decreased in the nervous system of mammals following a period of starvation, quantified by changes in phosphorylation of mTOR and downstream targets, including S6K. However, it was not known if dietary manipulations only of protein or amino acids would be sufficient to elicit similar changes in mTOR activity in the nervous system. Using the same downstream target of mTOR, S6K, as well as other measures of mTOR pathway activity, I show that the absence of dietary protein is correlated with reduced activity of the mTOR pathway and pathways under mTOR regulation. Moreover, removal of only dietary amino acids is sufficient to induce these changes. This suggests that neuronal mTOR activity does indeed respond to dietary protein and amino acid availability. Studies done in mice looking at starvation induced mTOR activity identified the mediobasal hypothalamus (MBH) of the brain as a region where changes in mTOR activity occurred [11]. It will be interesting in future studies to pursue brain imaging techniques in *Drosophila* to answer the question of whether the changes I see in mTOR activity markers are a result of changes in the whole brain or defined subregions, and if so, which subregions.

The dietary manipulations correlated with modified mTOR pathway activity are also sufficient to elicit changes in homeostatic feeding. This suggests these cellular changes in mTOR activity may be responsible for changes in neurons that can modify behaviour in a nutrient sensitive manner. In agreement with this hypothesis, genetic manipulations to inhibit and activate the mTOR pathway have previously been shown to modify food choice in adult *Drosophila* [7]. Following the inhibition

of mTOR activity in the nervous system by the over-expression of the mTOR inhibitors *TSC1* and *TSC2* the preference for yeast of male flies is enhanced, much as you would expect if inhibition of mTOR by lack of dietary protein would drive the homeostatic feeding. Of note, unpublished data from the laboratory shows that this enhanced yeast preference upon neuronal mTOR inhibition is not observed in flies that have been pre-fed a diet containing protein. Interestingly, no phenotype is observed in females following mTOR inhibition. This is unexpected given that activation of neuronal mTOR activity by the over-expression of *Rheb* gives a food choice phenotype in both males and females. It is possible the mTOR inhibition phenotype in females had been missed due to the ratiometric nature of the 2-choice colourimetric assay. In order to address whether mTOR activity is necessary in female flies for food choice behaviour I tested if the over-expression of the mTOR inhibitors *TSC1* and *TSC2* in amino acid fed and deprived females would yield changes in food choice of the flies. I used the flyPAD behavioural setup where the dynamic range of feeding is greater than the 2-choice colourimetric assay, in order to have a better chance of seeing a female phenotype. However, I found no evidence for enhanced yeast feeding in these flies. The only effect was a small reduction in sucrose consumption in the flies previously fed a diet with no amino acids. In conclusion, I found no evidence to support a *TSC1, TSC2* dependent mechanism driving food choice behaviour in amino acid deprived female flies. However, in future work it will be important to address whether protein and amino acid deprived flies respond differently to this genetic manipulation. Furthermore, it will be important to determine whether the *TSC1, TSC2* and *Rheb* dependent mechanisms perhaps represent

sexually dimorphic responses in flies in guiding food choice behaviour.

I also investigated components of the autophagy pathway, known to be under mTOR control, for a role in homeostatic feeding. I found tentative evidence for a role for *autophagy related gene 1* (*Atg1*) in the regulation of food choice. Loss of *Atg1* in the nervous system reduces yeast preference in these flies, following a period of protein deprivation. However, this gene also has an mTOR independent role in axonal transport, and this function could not be ruled out as contributing to the feeding phenotype observed.

The mTOR pathway, and those pathways under its regulation, such as autophagy are renowned for their high complexity and perhaps these genetic manipulations outlined above just lacked sufficient temporal or spatial specificity to highlight a role for this pathway in regulating protein appetite. Given the power of *Drosophila* genetics it is entirely possible to design future experiments taking this into account, and improve both the temporal and spatial specificity of such genetic manipulations.

7.1.3. *beefeater* is a novel neuronal gene regulating food choice behaviour

In an additional line of questioning I sought to determine whether the nervous system requires access to amino acids in order to effectively regulate feeding behaviour. It is known that amino acid sensing can take place both peripherally, along the gastrointestinal tract of the animal, and centrally in the nervous

system [12]. Neuronal amino acid sensing represents a very efficient way of linking nutrient availability to behavioural output. Using a novel sensor for free arginine I found changes in dietary protein and amino acids did seem to influence the levels of free arginine in neurons. Arginine is known to be a potent stimulator of mTOR activity, and so these fluctuations could indeed be driving the changes in mTOR activity seen following dietary manipulations. However, the relationship of these free arginine fluctuations with mTOR activity remains unclear. Whilst feeding a diet lacking protein seems to reduce free arginine levels, and this is consistent with decreased mTOR activity – feeding a diet lacking in amino acids increases free arginine in the cytoplasm, contradicting the decrease in mTOR activity seen. It has been shown that the lysosome is a key organelle for mTOR dependent amino acid sensing [13], so perhaps designing an arginine sensor targeted to this organelle, and quantifying free arginine levels at this intracellular site would be more informative in terms of describing mTOR associated activity. It is also possible that these fluctuations in amino acids drive changes in feeding behaviour independently of mTOR. Finally, further characterisation of the sensor in cell culture be required to determine the specificity of amino acid binding of the sensor and also the characteristics, both of which will help determine if the changes seen in sensor signal in the nervous system correspond to meaningful changes in amino acid levels.

If neuronal access to amino acids is necessary to drive homeostatic feeding behaviour then manipulation of the protein controlling this access should affect homeostatic feeding. I found that *CG12531*/*beefeater*, a highly conserved amino acid

transporter, localised to the lysosome, is both necessary and sufficient for controlling the set-point from which homeostatic yeast feeding behaviour is driven. *Beefeater* is conserved in mice and humans, where it is called SLC7A14, and where it is also found associated with the lysosome and transports cationic amino acids, including arginine, into the lysosome [14, 15]. The function of *beefeater* in controlling the set-point of homeostatic yeast feeding is intriguing. In amino acid fed flies expressing little or no *beefeater,* the set-point of homeostatic feeding is higher that that of wild type flies, and the flies eat more yeast. However, when challenged with a diet containing no protein or amino acids the mutant flies still respond by increasing yeast preference or intake even further. This suggests there are two parallel mechanisms, one controlling the set-point of homeostatic feeding that goes through *beefeater*, and another controlling responses in the face of dietary challenges.

I propose three possible models for the amino acid sensing mechanism of *beefeater* in neurons. In the first, *beefeater* acts as a buffering mechanism for cellular amino acids, and when there is less *beefeater* the steady-state levels of amino acids in the cytoplasm of the neuron, perhaps arginine, are low, setting a higher base-line of yeast feeding, as we see in the mutant flies. Conversely, when *beefeater* levels are high, as in the *beefeater* over-expressing flies, the steady-state of amino acids in neurons is also higher, setting a lower base-line of yeast feeding. When dietary manipulations reduce the levels of amino acids in the animal, higher or lower levels of *beefeater* can perhaps buffer the effect somewhat, but not enough to abolish the need to update the feeding behaviour. Indeed mutant flies show a robust

increase in yeast preference following yeast or amino acid deprivation. The second model speculates that *beefeater* may not act as a transporter, but as a 'transceptor', and in binding amino acids signal directly to a downstream pathway the availability of amino acids. In this model, it is the amount of amino acid bound *beefeater* that sets the set-point of homeostatic feeding. To prove either of these models, future experiments should aim to demonstrate the transport capabilities of *beefeater*. In order to test if *beefeater* transports amino acids, as has been shown for its mammalian homologue, or simply binds them, artificial expression in Xenopus oocytes followed by stimulation with various amino acids to determine flux capacity would need to be done. These experiments would also reveal the direction of transport – out of the lysosome or into the lysosome. Previous work has shown that multiple transporters can act synergistically to achieve gradients of different amino acids across cell membranes in order to drive the transport of other amino acids in the opposite direction [16]. Taking this into account, determining the direction of flux of *beefeater* may help further understand if *beefeater* acts alone or in combination with other amino acid transporters to exert its effects on behaviour. Furthermore, if arginine is one of the amino acids transported by *beefeater* the arginine sensor could also be used to determine the effects of *beefeater* on intracellular amino acid levels in neurons. The third model is one in which *beefeaters* function in regulating behaviour is independent of any amino acid transport or binding function. In this case *beefeater* may be responding to a nutrient dependent signal produced elsewhere in the fly, which is secreted into the haemolymph, travels to the nervous system where it exerts its effect on *beefeater*. Recently, a paper was

published describing a peptide, female–specific independent of transformer (FIT) [17], as a protein satiety signal. It is expressed in the adult head fat-body and promotes Dilp2 release from the IPCs. However, exactly how it achieves this remains unknown. Could *beefeater* be responding to FIT levels to guide yeast specific feeding behaviour?

In an attempt to understand what *beefeater* is doing in the neuron, and how it might be affecting feeding behaviour I addressed its effects on downstream pathways. I found no evidence to support a role for *beefeater* in modulating or genetically interacting with the mTOR pathway. In addition, I also found no evidence for beefeater regulating insulin release. These findings suggest that *beefeater* might represent part of an alternative cellular pathway playing a role in neurons in driving protein appetite.

However, determining what this alternative cellular pathway might be is not simple. In this case, the best strategy to find the affected downstream pathways may be to take an '-omics' approach. Comparisons of RNA, protein or metabolic products (RNAseq, proteomics or metabolomics) in both *beefeater* mutant flies and controls may uncover novel signalling pathways regulated by *beefeater.* Interestingly, the lysosome is capable of generating signalling molecules that can travel to the nucleus to activate transcriptional responses, or even to distant tissues to activate global homeostatic responses [13]. Perhaps *beefeater* acts upstream of one such signalling molecule. The second model I proposed above describes *beefeater* as a transceptor, and would suggest that *beefeater* may have direct binding partners that transduce the signal of when *beefeater* is bound to

amino acids. In this case co-immunoprecipitations could be used to find the binding partners of *beefeater.*

A surprising finding was the broad expression of *beefeater* in the nervous system. If *beefeater* is only required for the control of food choice it is striking that it should be expressed in so many neurons. Alternatively, *beefeater* may only function to regulate behaviour in some of the neurons it is expressed in, and fulfil other functions in the rest of the nervous system. In order to test this second possibility, I tried to find a subset of neurons in which *beefeater* manipulations were necessary and sufficient to modulate food choice. I failed to identify such a set of neurons. Leaving two hypotheses, *beefeater* is required in a very specific subset of neurons not covered in the battery of Gal4 lines I tested, or it really is required in all neurons, and through a global mechanism is able to control yeast feeding behaviour. It may be possible to distinguish between these two hypotheses with the following experiment: if all neurons are required, then the expression of *beefeater* IR pan-neuronally, in combination with the expression of any Gal80 (where the Gal80 protein inhibits the Gal4 driven UAS expression) that is expressed in the nervous system and therefore reduces the number of neurons in which *beefeater* is knocked down in should be sufficient to reduce the yeast feeding phenotype. However, the expression of the complimentary Gal4 to the Gal80 will not be sufficient to induce the feeding phenotype.

Finally, the discovery of a mutant that over-ingests specifically protein rich foods allows us to directly test the effects of such a diet on an animal's life history traits. For instance, evidence points to high protein diets limiting lifespan, but increasing

reproduction and vice versa. It will be interesting to determine if these effects hold true in *beefeater* flies. If *beefeater* flies eat more yeast in general they should also have shorter lifespans, and higher rates of reproduction.

To conclude, amino acid homeostasis is crucial to an animal's long-term health and wellbeing. My description of a new framework in which to define an amino acid balanced diet, and its effects on an animal's behaviour and life history traits confirms this importance. The regulation of feeding behaviour is one of the key mechanisms controlling amino acid homeostasis. The findings described in this thesis propose that mTOR activity is an important response in the nervous system to dietary amino acids, mediating transcriptional and degradation changes in the neuron to prepare the cell for nutrient stress. Furthermore, I identify a novel neuronal gene required for the regulation of homeostatic feeding behaviour, named *beefeater* **(Figure 7.1)**. This gene appears to regulate food choice through pathways independent of mTOR and insulin. The experiments I propose in this chapter should test whether direct access to amino acids by the nervous system plays a role in feeding behaviour, and provide a mechanism for how *beefeater* acts to control feeding behaviour. This would constitute a novel mechanism for the regulation of homeostatic feeding behaviour and amino acid homeostasis.

7.1

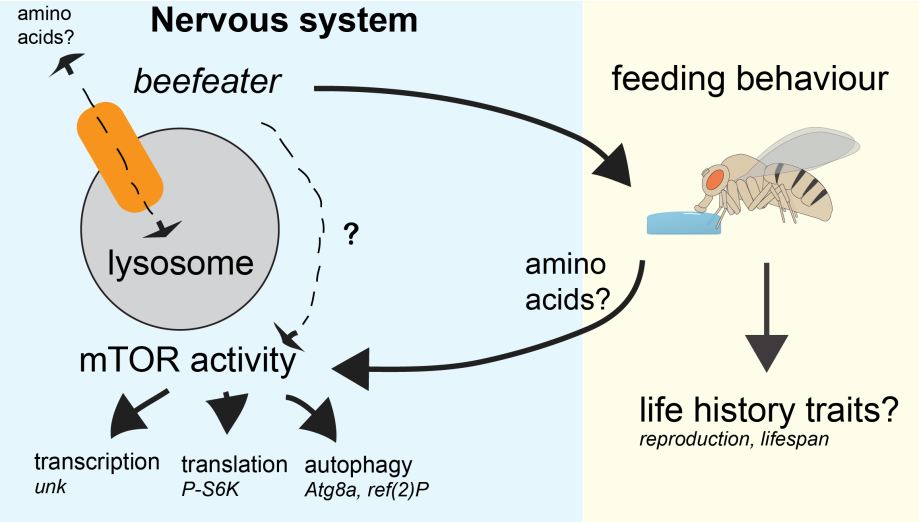


Figure 7.1. A schematic of the cellular mechanisms mediating amino acid homeostasis and food choice behaviour in female adult *Drosophila*. The model shows that mTOR activity is modulated in the nervous system according to the protein or amino acid composition of the food ingested. The modulation of mTOR pathway activity may be in response to fluctuating amino acids levels. Beefeater acts at the lysosomes of neurons, potentially transporting or binding to amino acids to regulate changes in food choice behaviour. *Beefeater* may be regulating food choice behaviour via neuronal mTOR signalling. The regulation of food choice by *beefeater* maybe have long lasting effects on the fly's life history traits such as reproduction and lifespan.

7.2. References

1. Payne, S. H. & Loomis, W. F. Retention and Loss of Amino Acid Biosynthetic Pathways Based on Analysis of Whole-Genome Sequences Retention and Loss of Amino Acid Biosynthetic Pathways Based on Analysis of Whole-Genome Sequences. *Eukaryot. Cell* **5,** 1–6 (2006).

2. Lee, K. P. *et al.* Lifespan and reproduction in Drosophila: New insights from nutritional geometry. *Proc. Natl. Acad. Sci.* **105,** 2498–2503 (2008).

3. Piper, M. D. W. *et al.* Matching Dietary Amino Acid Balance to the In Silico- Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan. *Cell Metab.* **25,** 610–621 (2017).

4. Lagniel, G. *et al.* Sulfur Sparing in the Yeast Proteome in Response to Sulfur Demand Mire. *Mol. Cell* **9,** 713–723 (2002).

5. Vijendravarma, R. K., Narasimha, S. & Kawecki, T. J. Predatory cannibalism in Drosophila melanogaster larvae. *Nat. Commun.* **4,** 1789 (2013).

6. Bjordal, M. *et al.* Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in Drosophila. *Cell* **156,** 510–21 (2013).

7. Ribeiro, C. & Dickson, B. J. Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. *Curr. Biol.* **20,** 1000–5 (2010).

8. Hao, S. *et al.* Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* **307,** 1776–8 (2005).

9. Leib, D. E. & Knight, Z. A. Rapid Sensing of Dietary Amino Acid Deficiency Does Not Require GCN2. *Cell Rep.* **16,** 2051–2052 (2016).

10. Itskov, P. M. & Ribeiro, C. The dilemmas of the gourmet fly: the molecular and neuronal mechanisms of feeding and nutrient decision making in Drosophila. *Front. Neurosci.* **7,** 12 (2013).

11. Cota, D. Hypothalamic mTOR Signaling Regulates Food Intake. *Sci. (New York, NY)* **312,** 927–930 (2006).

12. Fromentin, G. *et al.* Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutr. Res. Rev.* **25,** 29–39 (2012).

13. Mony, V. K., Benjamin, S. & O'Rourke, E. J. A lysosome-centered view of nutrient homeostasis. *Autophagy* **12,** 619–631 (2016).

14. Jaenecke, I. *et al.* A chimera carrying the functional domain of the orphan protein SLC7A14 in the backbone of SLC7A2 mediates trans-stimulated arginine transport. *J. Biol. Chem.* **287,** 30853–30860 (2012).

15. Jin, Z. *et al.* SLC7A14 linked to autosomal recessive retinitis pigmentosa. *Nat. Commun.* 1–9 (2014). doi:10.1038/ncomms4517

16. Nicklin, P. *et al.* Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. *Cell* **136,** 521–534 (2009).

17. Sun, J. *et al.* Drosophila FIT is a protein-specific satiety hormone essential for feeding control. *Nat. Commun.* **8,** 14161 (2017).

The work presented in this dissertation was carried out under the International Neuroscience Doctoral Programme (INDP), at the Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown under the supervision of Dr. Carlos Ribeiro, and the thesis committee supervision of Dr. Luísa Vasconcelos and Dr. Rui Costa. Financial support was given by a doctoral fellowship from Fundação para a Ciência e Tecnologia (SFRH/BD/76066/2011, attributed to Samantha Herbert)

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